CHEMICAL STUDIES OF ANTIBODIES AND OTHER SERUM GAMMA GLOBULINS

by

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I. INTRODUCTION

Immunological response, one of the most important factors in the defense of the organism against disease, is essentially the formation of antibodies. To understand this response one requires a knowledge of the mechanism of antibody formation. Before the conditions that control the formation of a new chemical compound can be determined, the characteristics of the molecule must first be established. This knowledge is achieved only after a careful study of the physical and chemical properties of the compound in comparison with those of known substances. When the chemistry of antibodies is understood, the mode of antibody biosynthesis may become capable of solution.

Although antibodies are now believed to be serum globulins, only thirty years ago the controversy concerning the nature of antibodies centered around the question of whether or not antibodies are proteins. Although several investigators, Fränkel (32), Olitzki (68) and others have claimed to have isolated non-protein antibodies, all attempts by other workers to confirm these results have been unsuccessful. By 1938 the evidence on behalf of the protein nature of antibodies was fairly conclusive. A comprehensive review by Marrack (56) emphasizes the following points. In the first place, it has not as yet been possible to separate the antibody activity of serum from the globulin fraction by any method of precipitation, or adsorption, or electrophoresis. Also, there is a very definite correspondence between the destruction of antibodies by heat, alcohol, or acid, and the denaturation of proteins by

these agents. Proteolytic enzymes such as pepsin and trypsin will destroy antibody activity. Several investigators (11,64) have observed that chemical modification of serum proteins reduces the activity of antibodies in the serum. Further evidence of the protein nature of antibodies has been derived from studies of antigen-antibody complexes. These precipitates always contain more protein than the antigen alone can account for. Felton (30), by dissolving the specific precipitate formed by antibody and the polysaccharide of Type I pneumococcus, obtained a protein which was 85 per cent reprecipitable with the specific polysaccharide. Even more striking evidence is found in the work of Heidelberger and Kabat (39,40). These workers observed that the total amount of protein nitrogen precipitated from an antipneumococcal horse serum by the specific polysaccharide is equal to the amount carried down by pneumococci of the same type when they are agglutinated by the antiserum. If the protein were being adsorbed non-specifically, along with a non-protein antibody, it would be extremely unlikely that the amount of adsorbed protein would be identical under these diverse conditions. Indeed, there remains little question that antibodies are serum globulins.

How antibody globulin differs from normal serum globulin is as yet unexplained. The electrophoretic properties, the chemical composition, the size and the shape of antibody and normal globulins have been compared by various investigators with the hope of solving this problem.

Electrophoretic studies of immune sera have shown that although antibodies may occur in any of the globulin fractions of serum, they are usually present in the γ-globulin fraction. Tiselius and Kabat (110), van der Scheer et al. (60,112,113) and Pappenheimer et al. (72) have analyzed immune globulin

fractions electrophoretically and have observed that the antibodies are present in the most slowly moving globulin fraction[1], the γ-globulin. More direct proof that the γ-globulin component is an antibody-containing fraction is obtained by comparing the electrophoretic pattern of immune serum with that of the same serum after the removal of antibodies by precipitation with the specific antigen. Tiselius and Kabat (110) observed that the removal in this manner of antibodies from immune sera of the rabbit and monkey produced a marked decrease in the γ-globulin content. Van der Scheer and his coworkers (113) reported a similar reduction in the amount of γ-globulin in antipneumococcal horse serum after removal of the antibody with the specific polysaccharide. In most antitoxic horse sera, however, a new globulin component with an electrophoretic mobility between the β- and γ-globulins, the T-globulin fraction, is evident and is found to carry the antibody activity alone or with the γ-globulin. Deutsch, Nichol and Cohn (26) have reported antibody activity associated with the α-globulin fraction of chicken serum. It is evident from these reports that antibodies may occur in any globulin fraction.

The distribution of antibodies in the various globulin fractions is complex. Smith and Gerlough (95) fractionated horse immune serum by alcohol precipitation techniques. They observed tetanus antitoxin to be present in at least three protein fractions: T-globulin, γ-globulin and a β-globulin fraction. In man most of the antibodies are found in Fraction II of Cohn

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[1] The conditions of electrophoresis suggested by Longsworth (55), namely, electrophoresis at pH 8.5 to 8.6 in a veronal buffer of 0.1 ionic strength, produces a separation of serum constituents into albumins, α-, β-, and γ-globulins, the latter being the slowest moving components.

et al. (20), a fraction which is mostly γ-globulin. However, it has been reported by Oncley (69) that typhi O antibodies and isoagglutinins are primarily found in Fraction III-1, a β-globulin.

Fractionation of the γ-globulin of several species by chemical and physical procedures has been described by many investigators (19,20,23,66,69). Such fractions can be further subdivided in the electrophoresis-convection apparatus of Kirkwood (50) into γ-globulins differing in electrophoretic mobility and isoelectric point. In other words, the γ-globulin of whole human serum appears to be a heterogeneous mixture of proteins. Nevertheless, Oncley et al. (69) have shown that in human γ-globulin the antibodies to several different antigens are equally distributed in Fractions II-1,2 and II-3, subfractions of Fraction II.

Recent studies of Cann and coworkers (17) have demonstrated that antibodies from the same animal with different specific combining affinities can be separated on the basis of a difference in their electrophoretic properties. Electrophoresis-convection was used in this study. A relative separation of the antibodies formed against the globulin portion of the antigen from those formed against the phenylarsonate derivative was achieved.

The isoelectric point of antibodies determined from electrophoretic data is usually within the range characteristic for normal γ-globulins, namely, pH 5.8 to 6.8. Lower isoelectric points of antibodies produced by horses, cattle and hogs against pneumococcus polysaccharides have been observed by several investigators; these studies have been summarized by Kabat and Mayer (48).

The studies mentioned in the preceding paragraphs, as well as many

others reviewed by Smith and Jager (98), indicate that certain antibodies in man and other species may be concentrated selectively in one or another subfractions of γ-globulin and at times even in other globulin fractions. Specific antibodies have also been found to be distributed throughout the serum globulin and not associated with any one serum component.

Much of the work previously reported on the chemical analysis of antibodies has been carried out on γ-globulin fractions of immune and normal sera. The amino acid and carbohydrate content of such globulin fractions isolated by alcohol precipitation from human, equine and bovine sera have been reported by Brand (8) and by Smith and coworkers (96,97). Their results, summarized by Smith and Jager (98), are reproduced in part in Table 1. These data are for certain globulin fractions, each fraction containing many antibodies. The complete amino acid composition of a purified specific antibody has not been determined. Although the proteins in Table 1 are very similar, species differences and differences between fractions of different electrophoretic mobility obtained from the same species are apparent.

Porter (80) has recently shown that normal rabbit γ-globulin and rabbit antiovalbumin each possess a single N-terminal residue, alanine and the same N-terminal peptide sequence, alanylleucylvalylaspartylglutamyl. The number of reactive ε-amino groups of lysine is identical for both preparations. These experimental data suggest that rabbit γ-globulin, unlike the γ-globulins of other species, is not a heterogeneous mixture of proteins of varying amino acid composition.

Considerable data have been obtained from ultracentrifugal studies concerning the size and shape of antibodies isolated from specific antigen-

TABLE 1

COMPOSITION OF IMMUNE PROTEINS

The data are given as gm. of constituent per 100 gm. of anhydrous protein. The values for bovine γ-globulin are averages for two preparations. The composition of human γ-globulin II-1 was reported by Brand (8). All other analyses except where specifically mentioned were performed by Smith and coworkers (96,97).

Constituent	Human γ-globulin II-1	Human γ-globulin II-1,2	Human γ-globulin II-3	Bovine γ-globulin	Bovine T-globulin	Equine γ-2-globulin	Equine T(γ-1)-globulin
Arginine	4.8	5.1	3.7	5.8	4.8	3.8	2.8
Aspartic Acid	8.8						
Cystine	3.1	2.6	2.7	2.9	2.8	2.6	2.5
Glutamic Acid	11.8						
Glycine	4.2						
Histidine	2.50	2.01	1.91	2.05	2.01	2.44	2.43
Isoleucine	2.7	2.8	2.0	3.2	3.0	4.4	3.3
Leucine	9.3	9.3	9.5	7.4	8.6	9.0	7.5
Lysine	8.1	7.2	6.3	6.7	6.4	8.6	6.7
Methionine	1.06	1.12	0.87	1.18	1.00	0.95	0.72
Phenylalanine	4.6	4.5	4.7	3.2	4.5	4.4	4.1
Proline	8.1						
Serine	11.4						
Threonine	8.4	8.8	7.4	10.0	9.5	11.1	8.7
Tryptophan	2.86	2.6	2.8	2.6	2.6	2.7	2.8
Tyrosine	6.75					6.8	6.8
Valine	9.7	9.7	9.7	10.0	9.5	10.1	10.4
Hexose		2.3	2.3	2.1	2.5	2.5	2.6
Hexosamine		1.27	1.23	1.31	1.50	1.18	1.53

antibody precipitates. Kabat (47), determining the molecular weights of various antipneumococcal antibodies from sedimentation data, found that rabbit and monkey antibodies have a molecular weight of about 160,000 to 180,000. This is the accepted molecular weight of normal γ-globulins. However, the bovine, equine and porcine antipneumococcal antibodies are much larger molecules having a molecular weight of 900,000. Pappenheimer and coworkers (72) found the molecular weight of equine diphtheria antitoxin to be 180,000. Several investigators (24,25,70,92,93) have demonstrated variable amounts of heavy components in γ-globulin and T-globulin fractions of high electrophoretic purity obtained from human, bovine and equine serum as well as from bovine colostrum and milk. Smith (92) and others have suggested that these heavy components are due to polymeric association of the lighter normal γ-globulin molecules. Cann and coworkers (16) from electrophoresis-convection studies of human, bovine and rabbit serum have also concluded that the heavier γ-globulins are artefacts formed during the alcohol fractionation procedures. As was previously mentioned, the specifically precipitated antipneumococcal antibody from horse serum appears to be composed entirely of the heavy γ-globulin. It seems essential that the pneumococcal antibodies isolated from horse antiserum by electrophoresis-convection and by specific precipitation be compared before the existence of a naturally occurring heavy γ-globulin can be excluded.

Ultracentrifugal and diffusion data indicate that both antibodies and normal γ-globulins are elongated in shape. Campbell and Bulman (15) have calculated the dimensions of several antibodies from frictional ratios. They report, for example, that the rabbit antibody against ovalbumin has the dimensions 244Å x 24Å.

Campbell and his associates (14) have determined the molecular weight of the rabbit antibody against p-phenylarsonic acid from light scattering data to be 160,000. This is in complete agreement with the value calculated from sedimentation and diffusion data.

In short, all properties of purified antibodies appear to be the same as those of normal serum globulins. Not only the amino acid composition of the antibodies, but also the molecular weight, the isoelectric point and other physicochemical properties are essentially the same as those properties of normal globulins. Although slight differences between normal and antibody globulins have been observed, similar differences exist between various globulin fractions of normal serum.

Antibodies and normal serum globulins are serologically distinct. Therefore, it is obvious that they must differ in some manner. In 1930 Breinl and Haurowitz (10,37) advanced the hypothesis that "the globulins are the true antibodies and that their antibody function is due to the complementary adaptation of their shape to the shape of the antigen". Similar hypotheses were advanced independently by Alexander (1) and Mudd (63). Since the strong determinant groups of antigen molecules are polar groups, it was assumed that their electrostatic forces directed the amino acid precursors of the protein into definite positions in such a way that the antibody so formed would be adapted in a manner complementary to the determinant group of the antigen. This could be achieved either by changes in the sequence of amino acids in the peptide chain or by a different mode of folding of the peptide chain.

Pauling (74) accepted this complementariness hypothesis and advanced

the theory that the difference between normal and antibody globulin is merely a difference in the way the polypeptide chain is folded or coiled. The basis of this theory is the assumption that antibody protein is synthesized in spatial contact with the determinant groups of the antigen. Pauling assumes that antibody globulin is synthesized first by the formation of a long uncoiled polypeptide chain that is identical with the precursor of normal globulin or any other antibody molecule. The chain is then folded to produce the configuration of greatest stability. The final structure is maintained by intramolecular hydrogen bonding. He assumes that for the central part of the chain there is one configuration that is more stable than any other. This structure would be common to normal globulin and antibody molecules. The two ends of the chain, on the other hand, can exist in many configurations of similar energy. When the folding process occurs with the two ends of the chain in contact with the antigen, the configuration which provides a complementary structure to the antigenic determinant group is adopted. This theory, commonly referred to as the Haurowitz-Mudd-Pauling theory of antibody formation assumes that the antigen is present in antibody-producing cells throughout the period of antibody production.

If antibodies differ from normal globulins only by variation in spatial configuration, it would seem possible that normal globulins could be converted to antibodies by an unfolding and refolding of their peptide chain in the presence of antigen. Pauling and Campbell (75) reported the production of antibodies <u>in vitro</u> by the action of mild denaturing agents on bovine γ-globulin and other serum proteins in the presence of certain antigens. These antibodies reportedly agglutinated the specific pneumococci and precipitated the specific polysaccharide. Haurowitz (37,38) later attempted

similar experiments with no success. The results obtained by Pauling and Campbell (75) were subsequently found to be due to nonspecific adsorption. Under controlled conditions of pH, the reversibly denatured γ-globulin caused no precipitation or agglutination (13).

Specific antibodies, being proteins, are themselves antigenic. Treffers and Heidelberger (11) immunized chickens with the specific precipitates of horse antiserum to pneumococcus Type I and II polysaccharide. They failed to find an antigenic difference although antigenicity is recognized as one of the most sensitive methods of detecting structural differences between protein molecules. Of course, because antigen-antibody precipitates were used as the antigens, the determinant groups or combining sites of the antibody were masked with polysaccharide. According to Pauling's hypothesis, the remainder of the molecules would be identical. This may expalin the observed results.

The fact that antibodies are not produced in vivo by a refolding of normal globulin is made clear by the experiments of Heidelberger et al. (44). When rabbits were injected with antibodies and N^{15} amino acids no uptake of N^{15} by the preformed antibodies was observed. This group of workers also showed that although antibodies are formed at the same rate as normal serum globulins they are not formed from these existing proteins.

The main opponent of Pauling's view that antigens play a direct role in antibody synthesis is Burnet. Although Burnet believes that antibody formation is initiated by the antigen, he believes that the production of certain antibodies can occur long after the antigen has disappeared from the organism. In order to explain the synthesis of antibodies in the absence of antigen, Burnet and Fenner (12) postulate that the antigen specifically modifies the

cellular enzymes necessary for globulin synthesis thus producing new enzymes with the ability to synthesize an antibody capable of reacting specifically with the antigen. They believe that these "adaptive enzymes" reproduce themselves even after the disappearance of antigen.

There is insufficient information about the chemistry of γ-globulins and pure antibodies to decide at present if either of these theories is correct in its entirety. The understanding of antibody formation necessitates a knowledge of the mechanism of protein synthesis. This in turn requires a knowledge of the amino acid sequence in the protein, the spatial configuration of the peptide chain and the position of attachment of prosthetic groups. Even with the techniques and analytical instruments available today, this detailed information is unattainable. However, it did seem possible that useful experimental data could be obtained. It has, therefore, been the purpose of this investigation to study the chemistry of several specific rabbit antibodies, as well as the chemistry of certain normal and immune globulin fractions of human, equine and bovine plasma. Also, because patients with multiple myeloma, a malignant disease of the plasma cells (considered by Fagraeus (20,29) to be responsible for antibody production) are known to produce abnormal globulins, certain myeloma proteins have also been investigated. By making use of the invaluable techniques of Sanger (86,87) to determine the sequence of amino acids at the N-terminal end of a peptide chain and by performing accurate amino acid analyses of specific antibodies by the method of Moore and Stein (62) it was hoped that controlled experimental data might be obtained supporting one or other of the two prevailing theories of antibody formation.

II. MATERIALS

A. 2,4-Dinitrophenyl-Amino Acids

The crystalline 2,4-dinitrophenyl-(DNP-) derivatives of glycine, L-alanine, L-valine, L-isoleucine, L-proline, DL-serine, DL-methionine, L-tryptophan and the di-DNP-derivatives of L-lysine, L-histidine, L-cystine and ϵ-DNP-L-lysine were prepared by Dr. N. C. Davis according to the method of Sanger described by Porter (82). DNP-L-aspartic acid, DNP-DL-glutamic acid, DNP-L-threonine, DNP-L-arginine, DNP-L-leucine and di-DNP-L-tyrosine were prepared in the same manner by the author and Dr. Davis. DNP-L-phenylalanine was prepared by Dr. A. Dannenberg by the standard procedure. Dr. E. O. P. Thompson supplied a sample of DNP-L-asparagine. The melting points of the crystalline compounds agree with those reported by Porter (82) and Rao and Sober (85). The 2,4-dinitrofluorobenzene (DNFB) used in the preparation of these reference compounds and the DNP-proteins was obtained from Eastman Organic Chemicals.

B. Rabbit γ-Globulin Solutions

Solutions of γ-globulins obtained from immune sera of rabbits immunized against Types I, II, III, IV, V, VII, VIII and XIV pneumococcal polysaccharides were given to Dr. E. L. Smith by E. R. Squibb and Sons. These solutions contain about 20 per cent protein. Approximately 20 per cent of the total

protein is specific antibody. Subsequently, a preparation of the total protein will be referred to as rabbit γ-globulin I and II, whereas, the specific antigen-antibody precipitates will be called rabbit antibody I and II.

C. Pneumococcal Polysaccharides

Types I, II, III, IV, V, VII, VIII and XIV pneumococcal polysaccharides, subsequently designated as SI and SII were also supplied by E. R. Squibb and Sons.

D. Human Serum Globulins

Human γ-globulins II-1,2 and II-3 were obtained from Dr. E. L. Smith. They have been previously characterized by ultracentrifugal, electrophoretic and amino acid analyses by Smith and coworkers (46,96,97). The amino acid composition of these is given in Table 1. The apparent isoelectric point of γ-globulin II-1,2 is pH 7.3, whereas that of γ-globulin II-3 is pH 5.85. No components other than γ-globulins were observed electrophoretically. Heterogeneity is indicated, however, because the electrophoretic patterns are broad and unsymmetrical. In the ultracentrifuge, both preparations were shown to contain about 75 per cent of a component with a sedimentation constant of about 6.5 to 7.0 Svedberg units and 25 per cent of heterogeneous heavier material sedimenting over a range of 9 to 17 Svedberg units. Both γ-globulins behaved as homogeneous antigens towards rabbit antisera. The two fractions appeared to be immunologically equivalent despite their known differences in other properties.

The "cryoglobulin" spontaneously precipitated from the cooled serum of a patient with multiple myeloma. The gummy white precipitate was washed several times with cold water. This crude preparation will be referred to as cryoglobulin A. A portion, cryoglobulin B, has been further purified by separation in an electrophoresis cell by D. M. Brown.

The two γ-globulins, myeloma globulins A and B, were isolated from the sera of two multiple myeloma patients by D. M. Brown. After the serum globulins had been precipitated with 50 per cent $(NH_4)_2SO_4$, the γ-globulins were further purified by electrophoresis-convection.

E. Bovine γ-Globulins

Bovine γ-globulins A and B were obtained from Dr. E. L. Smith and have been characterized by Smith and coworkers (91,96,97). γ-globulin A was obtained from sera of normal animals; γ-globulin B, from that of hyperimmune animals. The apparent isoelectric point of these γ-globulins is pH 7.2. In the ultracentrifuge 80 to 90 per cent of these electrophoretically homogeneous preparations have a sedimentation constant of 7.0 Svedberg units. The sedimentation constant of the heavier component is about 10.0 Svedberg units. The amino acid composition of these γ-globulins is given in Table 1.

F. Equine Serum Globulins

Equine γ-globulin and equine T-globulin were supplied by Dr. E. L. Smith. Their isolation and characterization have been described by Smith et al. (93,95,96,97). The preparation of equine γ-globulin, which is electrophoretically pure with its isoelectric point at pH 6.8, was reported to have an

antitoxic activity of 6,100 units per gram. The T-globulin, isoelectric point at pH 6.1, also had high antitoxic activity. The amino acid compositions are listed in Table 1.

Two preparations of the specific precipitate of horse antibody with Type III pneumococcal polysaccharide were supplied by Dr. M. Heidelberger. These preparations referred to as equine antibody III, were isolated from the sera of two different immune horses.

G. Constant Boiling Hydrochloric Acid

All hydrochloric acid was prepared by distilling a 20 per cent HCl solution in Pyrex glass three times.

H. Carboxypeptidase

A suspension of three times recrystallized carboxypeptidase from Worthington Biochemical Laboratories was treated with diisopropylfluorophosphate so that any trypsin or chymotrypsin present would be inactivated. The suspension of enzyme was assayed by Miss Anne Stockell.

I. DL-Glutamic Acid

The DL-glutamic acid used in the preparation of DNP-DL-glutamic acid (DNP-L-glutamic acid could not be obtained in crystalline form.) was prepared from L-glutamic acid by the method by Arnow and Opsahl (2).

J. Celite

Johns-Manville Celite Analytical Filter Aid #545 was used in the preparation of columns for chromatography of DNP-derivatives.

K. Ninhydrin

Ninhydrin or triketohydrindene, obtained from Dougherty Chemicals, was found to be sufficiently pure to be used without further recrystallization.

L. Dowex 50

Dowex 50-X8, 200 to 400 mesh, a cation exchange resin in the hydrogen form, was obtained from the Dow Chemical Company. This resin is a polystyrene sulphonic acid type resin of medium porosity.

III. EXPERIMENTAL METHODS

A. Physical Studies

1. Electrophoretic Analyses.

Electrophoretic analysis of several proteins was carried out in a Tiselius apparatus equipped with the Longsworth schlieren scanning device. The electrophoretic mobility and the amount of each constituent present was determined from the pattern produced by a descending migration at 1.5°. The protein solutions were equilibrated with pH 8.5 to 8.6 veronal buffer before analysis.

2. Ultracentrifugal Studies.

Sedimentation studies were performed in a Spinco[2] electrically driven ultracentrifuge at 59,780 R.P.M., equivalent to centrifugal fields of 240,000 x g and 300,000 x g at the meniscus and base. A description of this instrument and of the procedure followed in this laboratory has been published by Smith (94).

B. Immunological Studies of Rabbit γ-Globulins

1. Precipitin Reaction.

The amount of specific antibody in each rabbit γ-globulin solution

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[2] Specialized Instruments Corporation, Belmont, California.

and the composition of the specific precipitates were determined by the quantitative preciptin technique of Heidelberger and Kendall (42). A portion of the original γ-globulin solution was diluted with 0.9 per cent NaCl. Aliquots of this solution were mixed with increasing volumes of a 0.005 per cent solution of the specific polysaccharide and refrigerated for 5 days. Merthiolate solution, added to each tube, prevented bacterial contamination. During this time each tube was thoroughly mixed twice daily. After the mixtures had been centrifuged at a low temperature, the supernatant solutions were tested for excess antigen or antibody. The γ-globulin present in each precipitate was determined by a micro-modification of the Folin-Ciocalteu method described by Herriott (45). Human γ-globulin II-1,2 was used as the protein standard. A saline solution of the protein was prepared (approximately 1 mg. per ml.) and its exact concentration was determined by the procedure of Weichselbaum (114). This standard solution was diluted and used to standardize the Folin reagent. Later, the colour produced by a standard rabbit γ-globulin I solution (concentration obtained from Kjeldahl determinations using a value of 16.0 per cent for the nitrogen content) was compared with that of human γ-globulin II-1,2. Figure 1 demonstrates that no conversion factor is necessary when human γ-globulin is used as a standard for the measurement of rabbit γ-globulin.

2. Preparation of Specific Antigen-Antibody Complexes.

Several hundred milligrams of each antibody were precipitated at the equivalence point (Section IV-B,1) from the eight rabbit γ-globulin solutions by the addition of an appropriate amount of 0.005 per cent polysaccharide solution. The precipitates were separated by centrifugation from the super-

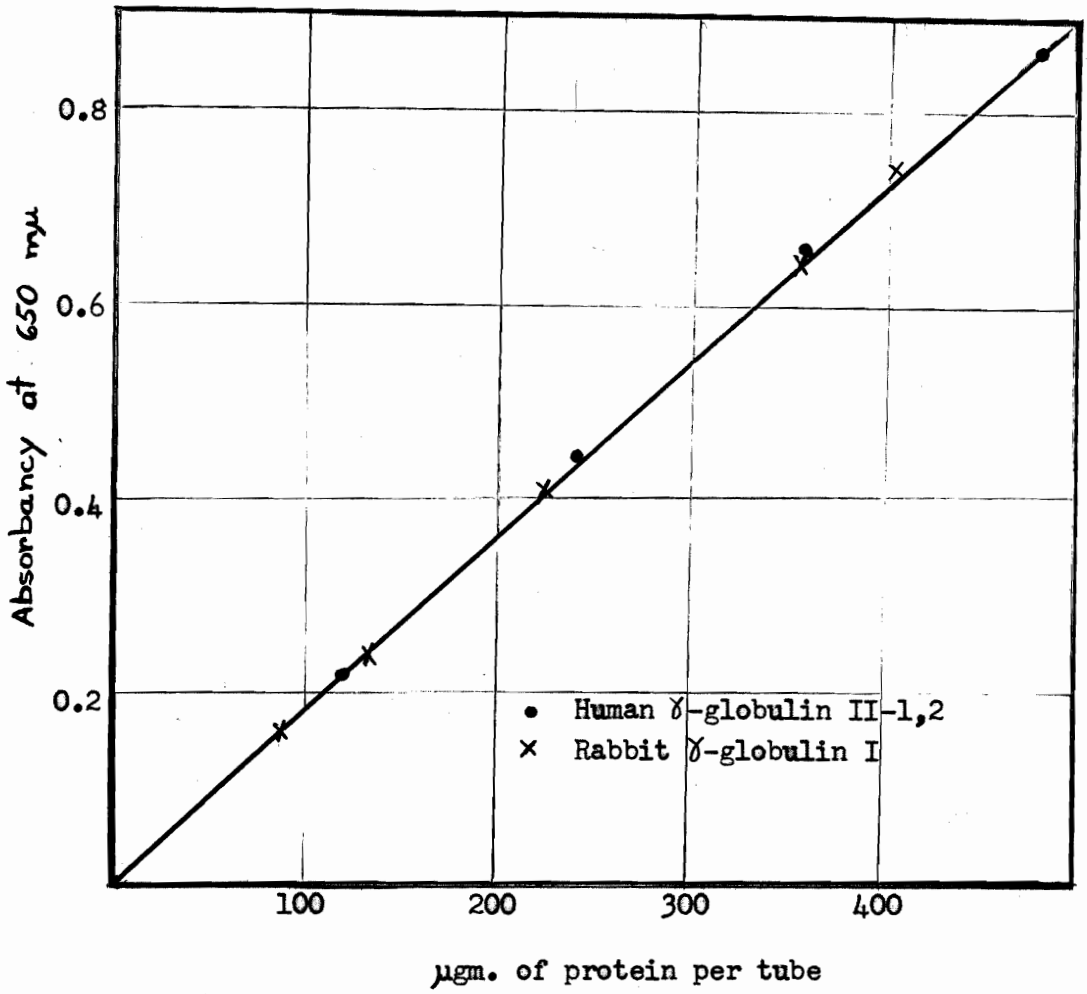

Figure 1. A Comparison of the Colour Produced by Human and Rabbit γ-Globulins with the Folin-Ciocalteu Phenol Reagent. The concentration of the human γ-globulin solutions was determined by the Biuret procedure (114); that of the rabbit γ-globulin solutions by a Kjeldahl nitrogen determination. (The nitrogen content of rabbit γ-globulin was taken to be 16.0 per cent). The colour was developed according to the method of Herriott (45).

natant solutions which were then tested for excess antigen and antibody. These precipitates, washed with saline, deionized water, absolute ethanol and anhydrous ethyl ether, were allowed to equilibrate in air at room temperature for 2 days.

It later became necessary to determine if free amino acids, especially aspartic acid, were being bound to the γ-globulin molecule or to the antibody-antigen complex. For this reason, a second preparation of Type III antibody was precipated by the specific polysaccharide in the presence of 3 equivalents of L-aspartic acid. This precipitate was washed and treated as any other. It has been called rabbit antibody IIIa in the discussion to follow.

3. Isolation of Other Rabbit γ-Globulins.

The protein remaining in γ-globulin solution VII after precipitation of the antibody with Type VII polysaccharide at the equivalence point (no antigen or antibody remaining in solution) was precipitated with alcohol and washed as previously described. This preparation is referred to a γ-globulin-VII. Similarly, γ-globulin M is a mixture of γ-globulin-VIII and γ-globulin-XIV.

Normal whole rabbit γ-globulin N was isolated from freshly clotted rabbit blood according to the procedure of Kekwick (49). This preparation was shown by electrophoresis to be 95 per cent γ-globulin.

4. Dissociation of Specific Precipitates with $Ba(OH)_2$.

An unsuccessful attempt was made to dissociate the specific precipitates or rabbit antibodies II and IV with $Ba(OH)_2$ by the method of Heidelberger et al. (41,43). Denaturation of the antibody protein under these conditions is great and no purified antibodies were recovered.

C. Preparation of 2,4-Dinitrophenyl-Proteins

1. Reaction of Protein with 2,4-Dinitrofluorobenzene.

2,4-Dinitrofluorobenzene (DNFB) reacts with the free amino, imino, phenolic hydroxyl, thiol, imidazole and quanidino groups of proteins. When a dinitrophenyl-protein (DNP-protein) is hydrolyzed in acid the dinitrophenyl bonds are cleaved much less readily than are the peptide bonds of the protein. Therefore, a number of DNP-amino acids can be isolated from an hydrolysate. These DNP-amino acids correspond to amino acid residues in the protein which possess functional groups not involved in chemical bonds. For example, the ϵ-amino groups of lysine and the α-amino group of an N-terminal amino acid become substituted when a protein is treated with DNFB.

DNP-proteins were prepared according to the method of Sanger (82,86). The protein and an equal weight of $NaHCO_3$ were dissolved or suspended in water. To every ml. of solution or suspension (approximately pH 8.5) 2.0 ml. of DNFB solution (10.0 of DNFB per 100 ml. of absolute ethanol) were added. This mixture, after being shaken vigorously in the absence of light for 3 hours, was centrifuged and the DNP-protein, a yellow powder, was washed thoroughly three times each with deionized water, absolute ethanol and ethyl ether and allowed to equilibrate in air for at least 48 hours.

All DNP-derivatives unless otherwise specified were prepared in this manner.

2. Control Experiments.

a. The effect of pH and time on the completeness of reaction. The DNP-preparations designated human γ-globulin II-1,2 B and II-3 B were prepared in the standard manner with one exception. The time of shaking was increased

from 3 to 16 hours. DNP-rabbit γ-globulin M was prepared by treatment with DNFB for 24 hours. These experiments were carried out to determine if a greater number of amino groups became substituted when the protein was left in contact with DNFB for a longer period of time. Also, in order to determine the effect of pH on the extent of reaction, human γ-globulin II-3 C and rabbit γ-globulin I B were treated with alcoholic DNFB in the presence of pH 6.5 phosphate buffer rather than a solution of $NaHCO_3$ (pH 8.5). Human cryoglobulin B in pH 8.5 veronal buffer, was treated with twice the volume of DNFB solution. No $NaHCO_3$ was added.

b. The effect of reaction time on the stability of DNP-protein. Because Weygand and Junk (115) and Thompson (108) have reported that some breakdown of protein occurs during treatment with DNFB, the supernatant solution and all the washings of some preparations were collected and examined for free DNP-amino acids. The ether and ethanol were removed at reduced pressure and the basic solution remaining was extracted with ether to remove any unreacted DNFB, then acidified and any acidic DNP-amino acids present were extracted into ether. These ether extracts were taken to dryness and chromatographed on buffered Celite columns and paper as is described in Section III-D, 3a.

c. The presence of free amino acids in γ-globulin solutions. Because there was the possibility of some hydrolysis of the protein during storage by bacterial proteolytic enzymes, an aliquot of rabbit γ-globulin solution, Type I, was shaken for 1 hour with the acid form of Dowex 50 resin. The protein solution was decanted off and the resin washed thoroughly. Amino acids, if present, were eluted from the resin with concentrated ammonia. The solution, placed on a polythene strip, was evaporated to dryness in vacuo

and the residue then dissolved in distilled water, chromatographed and developed as will be described later. A general assortment of amino acids were present but in total amount not exceeding 250 µgm. per ml. of solution. This amount represents negligible interference in the experiments so far described as well as in subsequent descriptions.

d. Reaction of DNFB with pneumococcal polysaccharides. Samples of Types I, III and VIII pneumococcal polysaccharide were treated with DNFB and the DNP-derivatives isolated and hydrolyzed. The hydrolysates were extracted and both the ether extracts and the acid fractions were chromatographed on buffered Celite columns. Aliquots of both fractions were chromatographed on paper using buffered tertiary amyl alcohol (Section III-D, 36). This experiment was thought necessary because the eight preparations of rabbit antibodies each contained 2 to 6 per cent specific polysaccharide. However, no coloured compounds were produced.

3. Composition of DNP-Proteins.

The amount of moisture-free protein in the air-equilibrated DNP-proteins was determined by estimating the amide content of the air-dried proteins and the DNP-preparations and the moisture content of the air-equilibrated proteins.

a. Amide ammonia determination. Amide determinations were carried out according to a modification of the method of Bailey (3). Accurately weighed samples were heated with 2 N HCl in sealed tubes at 105° for 4 hours. The solutions were quantitatively transferred to a Kjeldahl distilling vessel and neutralized to pH 5 or 6 (bromocresol green) with 6 N NaOH. The ammonia liberated after the addition of pH 10 to 11 phosphate buffer (15 ml. of 0.1 M

NaOH added to 50 ml. of 0.15 M Na_2HPO_4 solution) was steam distilled for 4 minutes into 0.01 N standard HCl. Excess acid was determined by back titration with 0.01 N standard NaOH using Tashiro's indicator. This indicator was used because CO_2, if present, causes no interference with the end point. The preparation of the indicator is described by Cole (21). Reagent blanks were analyzed with every group of duplicate determinations.

b. Moisture determination. The moisture present in the air-equilibrated proteins was calculated from the weight lost by an accurately weighed sample after it had been heated at 105° to constant weight.

D. The Free Amino Groups of Serum Globulins

1. Hydrolysis of DNP-Proteins.

The DNP-proteins were completely hydrolyzed by heating at 105° with 6 N HCl in sealed Pyrex tubes for 16 to 25 hours. Because DNP-proline and DNP-glycine are destroyed under these conditions, additional hydrolyses were carried out. A sample of DNP-rabbit γ-globulin M was heated with 12 N HCl at 105° for 24 hours. Under these conditions approximately 30 per cent of the DNP-glycine and DNP-proline should be recovered. Another sample of the same preparation was hydrolyzed for 4 hours with 6 N HCl at 105°. A 50 per cent recovery of DNP-glycine should be obtained under these conditions. A sample of DNP-bovine γ-globulin A was hydrolyzed with 6 N HCl for 4 hours to establish the presence or absence of DNP-glycine. These methods of Sanger have been summarized by Porter (82).

2. Extraction of Hydrolysates.

The acid hydrolysates were diluted to a concentration of approximately 1 N HCl and extracted four or five times with equal volumes of ether. These ether extracts, washed three times with small volumes of distilled water, were concentrated to dryness under reduced pressure at 50 to 60°. The residues were dissolved in acetone and reevaporated to remove HCl and water. Most of the dinitrophenol produced from the destruction of DNP-derivatives during hydrolysis was removed by sublimation at low pressures onto a "dry ice" finger by the method of Mills (53). The acid fraction and the washings were combined and diluted to a known volume. (ϵ-DNP-lysine, di-DNP-histidine, N^1-DNP-arginine, O-DNP-tyrosine and S-DNP-cysteine, if present, remain in the acid fraction). A suitable aliquot was evaporated under reduced pressure. HCl was removed by washing down the residues with acetone and again evaporating.

3. Separation and Identification of DNP-Amino Acids.

a. Chromatography of DNP-amino acids on buffered Celite columns. A combination of the methods described by Blackburn (6), Perrone (76), Bailey (4) and Middlebrook (57) was used.

Preparation of columns. Ten mg. of Celite #545 was ground in a mortar with 6.0 ml. of the desired buffer solution. This buffered Celite could be stored for two weeks in a tightly sealed jar. Columns were prepared immediately before use from a mixture buffered Celite and a suitable water-saturated solvent as described by Thompson (109). The following buffer solutions were used in the preparation of columns:

1. pH 4.0 — 1 M NaH_2PO_4.
2. pH 5.0 — 21 gm. of citric acid per liter of 0.2 M NaOH.
3. pH 5.5 — 1 ml. of 0.25 M Na_2HPO_4 to 9 ml. of 0.25 M NaH_2PO_4.
4. pH 6.5 — 5 ml. of 0.25 M Na_2HPO_4 to 5 ml. of 0.25 M NaH_2PO_4.
5. pH 7.1 — 8 ml. of 0.25 M Na_2HPO_4 to 2 ml. of 0.25 M NaH_2PO_4.

The following water-saturated solvents have been found to be the most useful for the separation of DNP-derivatives on buffered Celite columns:

1. CE 50 — 50 ml. of chloroform to 50 ml. of ethyl ether.
2. $CHCl_3$ — Water-saturated chloroform was used in the preparation of solvent systems or itself as a solvent.
3. CB 2 — 2 ml. of normal butanol to 98 ml. of chloroform.
4. CB 15 — 15 ml. of normal butanol to 85 ml. of chloroform.
5. EtAc — Water-saturated ethyl acetate.
6. Ether — Water-saturated ethyl ether. This solvent, stored over a ferrous sulfate solution to prevent the formation of peroxides, evaporated too readily on warm days to be an ideal solvent.

Chromatography of acidic DNP-amino acids. The residues of the ether extracts (Section III-D,2) were dissolved in a minimum amount of CE 50 and quantitatively transferred to the surface of a pH 4.0 CE 50 column. Development of the yellow band with CE 50 effects a separation of DNP-aspartic acid (R= .07)[3], DNP-serine (R = .23), DNP-glutamic acid (R = .35) and DNP-threonine (R = .6). All other compounds travel more rapidly (R > 1.0) on this solvent-column system. If a good separation of DNP-serine

[3] R is defined as the ratio of the distance travelled by the band in the column to the distance moved by the solvent above the column.

and DNP-glutamic was not obtained on this column, the mixed band was resolved on a pH 5.5 EtAc column (DNP-glutamic acid, R= .10; DNP-serine, R = .35). The fast band from the pH 4.0 column was then taken to dryness and dissolved in the minimum amount of $CHCl_3$ and transferred to a pH 6.5 $CHCl_3$ column. On this column DNP-alanine and DNP-proline (R = .02), DNP-valine and DNP-methionine (R = .25), DNP-phenylalanine and di-DNP-lysine (R = .56) are separated from DNP-leucine, DNP-isoleucine, dinitrophenol and dinitroaniline (R > 1.0). DNP-glycine and di-DNP-tyrosine which do not move (R=0) can be eluted from this column with ether, ethyl acetate or acetone. The fast band from the pH 6.5 $CHCl_3$ column is chromatographed next on a pH 7.1 $CHCl_3$ column which will distinguish DNP-leucine and DNP-isoleucine (R= .15 to .25) from dinitrophenol (R= .45) and dinitroaniline (R=1.0). Porter (82) has described solvents which separate pairs of DNP-amino acids with the same R values if both are present in the hydrolysate.

Chromatography of basic DNP-amino acids. ϵ-DNP-lysine in the residues of the acid fraction (Section III-D,2) was purified on a 1 N HCl column using CB 15 (R = .26). The acid fractions of DNP-human γ-globulin II-1,2, II-3, DNP-bovine γ-globulin A, DNP-rabbit γ-globulin I and DNP-rabbit antibodies III and XIV were chromatographed by the sensitive method of Bailey (4) to identify basic DNP-residues other than ϵ-DNP-lysine.

b. Chromatography of DNP-amino acids on paper. The R value of a yellow DNP-derivative on a Celite column cannot be used as absolute identification of the compound. R values vary with the packing of the column, the amount of DNP-derivatives on the column and the composition of the water-saturated solvent which varies with temperature. For this reason,

each band, after quantitative estimation (Section III-D,4), was chromatographed on paper for positive identification. Two to 10 µgm. of DNP-amino acid dissolved in acetone are applied 2.5 or 3 inches from the top of a 56 cm. strip or sheet of suitable filter paper. The paper is then fixed in a glass trough mounted near the top of a tall cylindrical glass jar and allowed to come to equilibrium with the solvent- and water-saturated air within the closed jar. Water-saturated solvent, placed in the trough is allowed to travel down the paper to the lower edge. If a further separation of spots is desired, the paper is "pinked" and the solvent allowed to drip off into the bottom of the chromatography jar. The following solvents have been found to effect good separation of DNP-amino acids and to produce round, compact spots.

Tertiary amyl alcohol. Blackburn and Lowther (7) have found that tertiary amyl alcohol (TAA) saturated with pH 6.0 phthalate buffer (45.40 ml. 0.2 M NaOH added to 50 ml. of 0.2 M potassium acid phthalate and the resulting solution diluted to 200 ml.) effects a good resolution of DNP-amino acids if they are chromatographed on paper which has previously been soaked in pH 6.0 phthalate buffer and dried at room temperature. Such chromatograms are usually run for 3 to 5 days on "pinked" Whatman #1 paper. The approximate position of the DNP-amino acids after development with this solvent is shown in Figure 2. This system does not separate DNP-aspartic acid and DNP-glutamic acid, DNP-serine and DNP-glycine, DNP-alanine and DNP-proline, DNP-valine and DNP-phenylalanine or DNP-leucine and DNP-isoleucine.

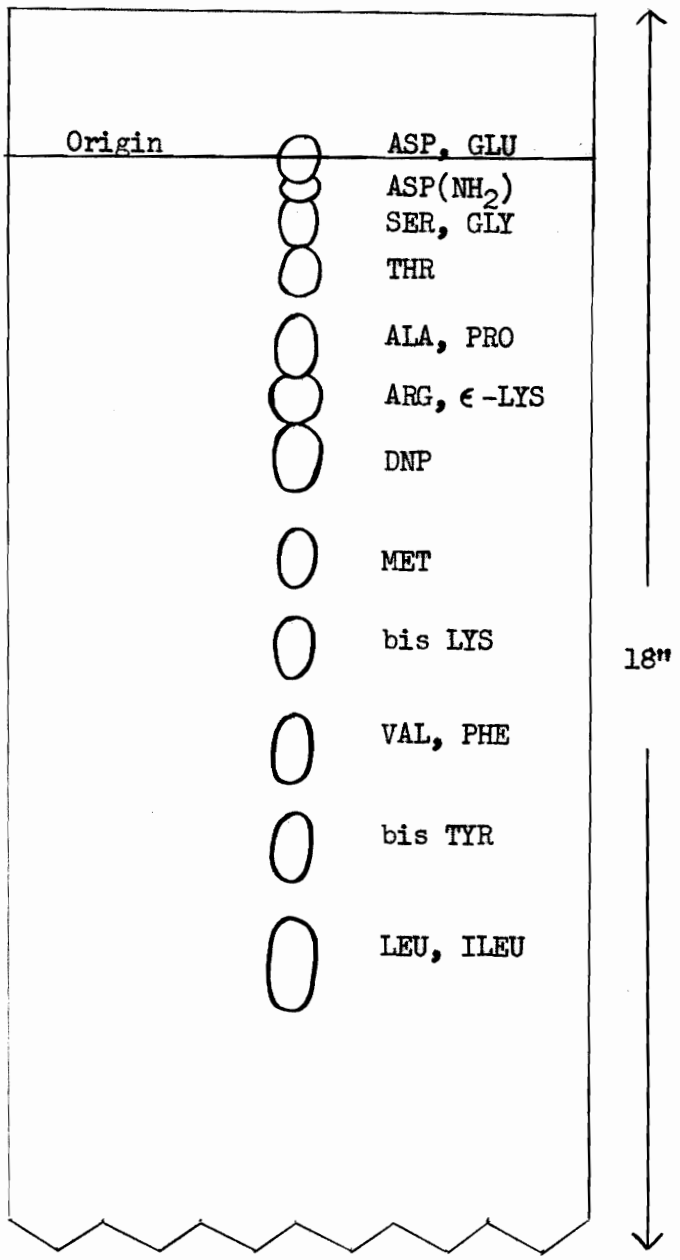

Figure 2. The Approximate Position of DNP-Amino Acids on Buffered (pH 6.0) Whatman #1 Paper After Development With Buffered (pH 6.0) Tertiary Amyl Alcohol for 5 Days.

The abbreviations for the amino acids used in Figure 2 and elsewhere in this paper follow the suggestions of Brand and Edsall (9) and Sanger (88). DNP= dinitrophenol.

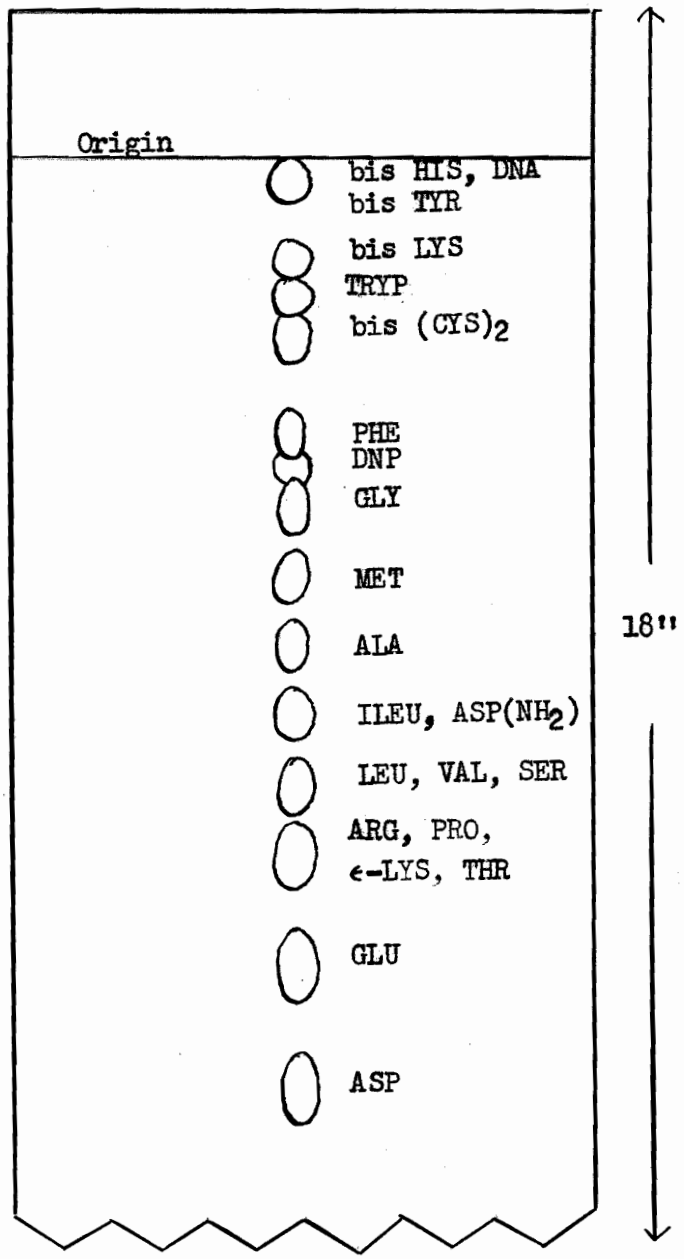

Figure 3. The Approximate Position of DNP-Amino Acids on Whatman # 1 Paper After Development with 1.5 M Phosphate Buffer for 24 Hours.

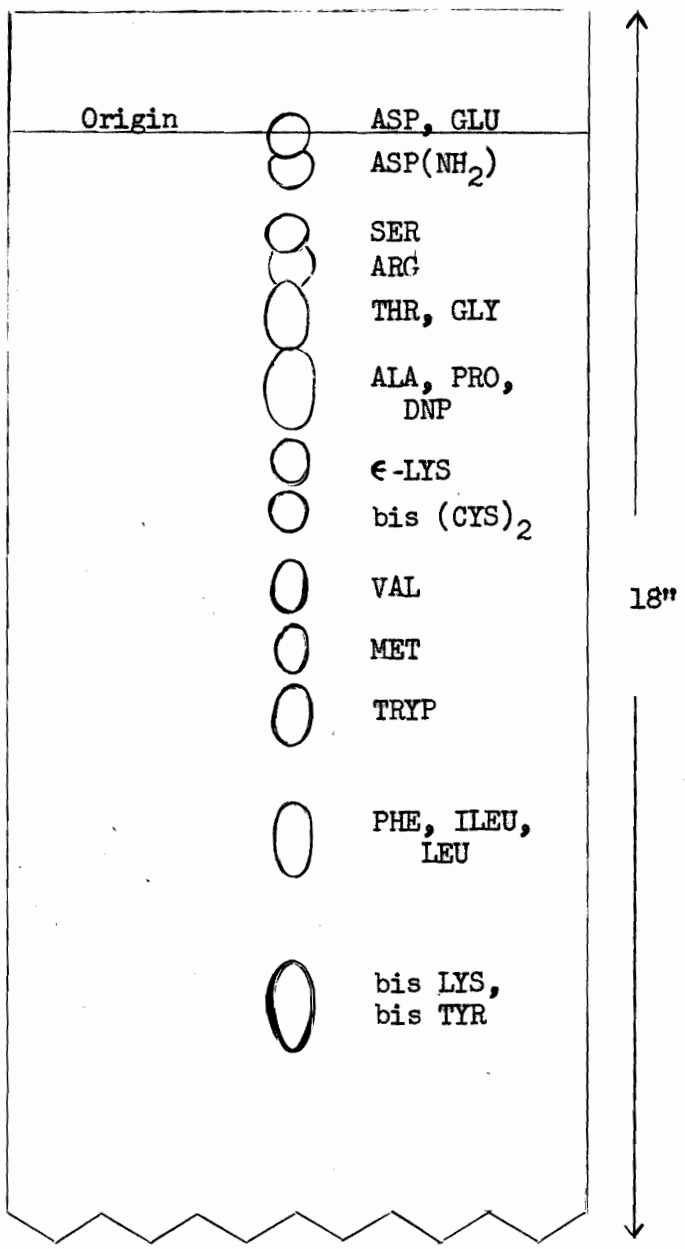

Figure 4. The Approximate Position of DNP-Amino Acids on Whatman #1 Paper After Development With the "Toluene System" for 15 Hours.

1.5 M phosphate buffer. Levy[4] has obtained good resolution in 24 hours of DNP-amino acids on Whatman #1 paper developed with 1.5 M NaH_2PO_4 solution adjusted to pH 5.9 to 6.0 with 1 M NaOH. The approximate position of the DNP-amino acids after 24 hour development is shown in Figure 3. This system is useful for the identification of DNP-aspartic acid, DNP-glutamic acid, DNP-alanine and DNP-proline. It also distinguishes DNP-valine and DNP-phenylalanine.

Toluene. Biserte (5) developed a solvent system useful for the separation of DNP-serine and DNP-glycine. A good separation of ϵ-DNP-lysine, DNP-proline and DNP-arginine is also achieved using this system. Five volumes of toluene, 1 volume of pyridine and 3 volumes of ethylene chlorohydrin are mixed with shaking. Three volumes of 0.8 N NH_4OH are then added without mixing and the two phases are allowed to equilibrate for 1 hour. The aqueous phase and an equal volume of 0.8 N NH_4OH are placed in the bottom of the chromatography jar. The organic phase, filtered through Whatman #1 paper, is used to develop the chromatogram. Good separation is complete in 12 hours. The disadvantage of this system is its instability. Fresh solvent must be prepared every 3 to 5 days. The positions of the DNP-amino acids are shown in Figure 4.

c. Recovery of amino acid from DNP-amino acid. Three samples of the DNP-derivative obtained from hydrolysates of DNP-rabbit preparations which appeared to be DNP-aspartic acid (Table 21) were pooled (DNP-rabbit antibodies II, V and VIII). The combined $NaHCO_3$ solution was acidified

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[4] Private Communication from E. O. P. Thompson concerning the unpublished technique of A. L. Levy.

and extracted with ether. The residue from the washed ether extracts was dissolved in saturated $Ba(OH)_2$ solution. This solution, sealed in a thick walled Pyrex tube, was heated for 2 hours at 140°. The Ba^{++} was removed from the cooled solution by the addition of a few crystals of "dry ice". The mixture was centrifuged and the clear supernatant solution evaporated *in vacuo* on a polythene strip. The residue, dissolved in one drop of water, was chromatographed on Whatman #1 paper using phenol as a solvent as will be described in Section III-E,6. This method for the regeneration of amino acids from their DNP-derivatives was first suggested by Mills (58). DNP-leucine was also confirmed by this method. Leucine was distinguished from isoleucine on tertiary amyl alcohol chromatograms (Section III-E,6).

4. Quantitative Estimation of DNP-Amino Acids.

Single yellow bands eluted from the buffered Celite columns were taken to dryness *in vacuo* and dissolved in a known volume of solvent. The acidic DNP-amino acids were dissolved in a 1 per cent solution of $NaHCO_3$; ϵ-DNP-lysine was dissolved in 1 N HCl. Sanger (87) has reported that the absorption maximum of a $NaHCO_3$ solution of DNP-glycine is 350 mμ. The absorbancy index,[5] calculated from his data is 0.0155×10^6 moles^{-1} liter cm^{-1}. He suggests that ϵ-DNP-lysine in 1 N HCl should be estimated at 390 mμ because of the interference of O-DNP-tyrosine at 360 mμ, the absorption maximum of ϵ-DNP-lysine. The absorbancy index of ϵ-DNP-lysine calculated from Sanger's data at 390 mμ is reported to be 0.0102×10^6 moles^{-1} liter cm^{-1}. [5]

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[5] The absorbancy (A) of a solution is defined as -log T where T is the transmittancy of the solution. $A = abc$ where a = absorbancy index; c = concentration of solution in moles per liter; b = width of cell in cm.

The absorbancies[5] of 1 M HCl and 1 per cent $NaHCO_3$ solutions of DNP-amino acids in 1 cm. matched Corex cells were read in a Beckman Model DU Spectrophotometer against a blank of either 1 M HCl or 1 per cent $NaHCO_3$.

a. Absorption spectra and absorbancy indices. Standard solutions of pure DNP-L-aspartic acid, DNP-DL-glutamic acid, and DNP-L-alanine in 1 per cent $NaHCO_3$ were prepared and the absorbancy of each from 320 mμ to 450 mμ at 2 mμ intervals was determined. Similarly, the absorption spectrum for a standard solution of ε-DNP-lysine in 1 N HCl was determined. The absorbancy index of the DNP-derivatives of L-valine, L-isoleucine and DL-serine was determined from the absorbancy of 1 per cent $NaHCO_3$ solutions of each compound at 350 mμ.

b. Recovery of DNP-amino acids after acid hydrolysis. The recovery values of DNP-amino acids determined by Sanger and Porter (89) are given in Table 2. These values have been used in subsequent calculations. No attempt was made to determine the actual breakdown of DNP-amino acids. Fraenkel-Conrat and Porter (31) and Middlebrook (57) have published recovery values which agree fairly well with those reported by Sanger and Porter (89). Thompson (109) has shown the destruction of DNP-amino acids to be due to tryptophan and cystine.

E. N-Terminal Sequence of γ-Globulins

1. Partial Hydrolysis of DNP-Proteins.

Certain of the DNP-proteins were partially hydrolyzed either by heating with 6 N HCl in a sealed tube for 1 to 2 hours or by incubating at 37° with 12 N HCl in a stoppered flask for 4 to 8 days.

TABLE 2

APPROXIMATE BREAKDOWN OF

DNP-AMINO ACIDS DURING ACID HYDROLYSIS.

This table is in part derived from data presented by Sanger and Porter (89).

Amino acid derivative	Percent recovered	
	18 hours	24 hours
DNP-aspartic acid	70	60
" glutamic "	66	56
" serine	85	80
" threonine	92	90
" alanine	70	60
" valine	70	60
" leucine	70	60
" isoleucine	70	60
" lysine	92.5	90

2. Extraction of Partial Hydrolysates.

Because some DNP-peptides are only slightly soluble in ether, the partial hydrolysates were diluted to 10 times their volume with water and extracted four times with equal volumes of ethyl acetate. This procedure of Sanger (87) also extracts a great many ϵ-DNP-lysine peptides which can be partially removed as follows: The ethyl acetate extracts, washed three times with small amounts of water, were extracted three times with one-quarter

the volume of 1 per cent $NaHCO_3$. The ethyl acetate-washed $NaHCO_3$ solution was then acidified and extracted three times with ethyl acetate. These extracts, washed with small amounts of 1 N HCl, were evaporated to dryness under reduced pressure at temperatures below 30°.

3. Isolation of DNP-Peptides on Buffered Celite Columns.

The same buffered columns and group of solvents used to separate DNP-amino acids were used to separate DNP-peptides. The schematic separation of each of the six partial hydrolysates into DNP-peptides and DNP-amino acids is shown in Figures 15 to 18 in Section IV-H.

4. Hydrolysis of DNP-Peptides.

Each eluted band was dissolved in 1 per cent $NaHCO_3$ and the absorbancy of the solution was determined at 350 mμ as described in Section III-D,4. An arbitrary value, $.0156 \times 10^6$, for the absorbancy index of all peptide solutions was used. No correction factors for breakdown of the DNP-derivatives were employed in the calculation of amounts of DNP-peptides isolated. The bicarbonate solution was then acidified and the DNP-derivative was extracted into ethyl acetate. The extracts were washed twice with small amounts of water to remove free amino acids and then taken to dryness in vacuo. The residue was dissolved in 3 to 6 drops of 6 N HCl with warming, sealed in a capillary tube, and heated to 105° for 20 hours.

5. Fractionation of DNP-Peptide Hydrolysates.

The cooled peptide hydrolysate was transferred to a small tube and thoroughly extracted with ether. The ether extracts were taken to dryness and chromatographed on paper as described in Section III-D,3b. The ex-

tracted hydrolysate, freed of dissolved ether under reduced pressure was transferred to a polythene strip and taken to dryness in a vacuum dessicator. When dry and acid-free, the residues were dissolved in a minimum amount of water and applied with a capillary pipette in a small spot (0.5 cm.) to Whatman #1 paper. Hot air was used to dry the spot as the solution was applied. The paper chromatography of amino acids will be discussed in Section III-E,6. If, after ether extraction, the DNP-peptide hydrolysate remained yellow, an aliquot of the extracted hydrolysate was chromatographed on #1 paper with either buffered tertiary amyl alcohol or toluene (Section III-D,3b) to establish the presence of ϵ-DNP-lysine.

6. Identification of Amino Acids by Paper Chromatography.

Small spots, 0.5 cm. in diameter, were applied 3 inches from the top of a 20 inch sheet of Whatman #1 paper. The amino acids moved more quickly on #4 paper; however, the spots were diffuse and tailed badly. The chromatograms, after development with solvent, were allowed to dry at room temperature. Phenol was removed by thoroughly washing the chromatograms with ether. The dry chromatograms were then sprayed with ninhydrin reagent and slowly heated while still wet until the coloured spots developed.

a. Butanol-acetic acid. This solvent first described by Partridge (73) consists of 4 volumes of normal butanol to 5 volumes of water to 1 volume of glacial acetic acid. When the bottom edge of the paper is "pinked" and the solvent allowed to drip off for 40 hours, a good separation of all amino acids except leucine and isoleucine, valine and methionine, glutamic and threonine, serine and glycine, lysine and arginine is achieved. Figure 5 shows the approximate positions of the amino acids after development on Whatman #1

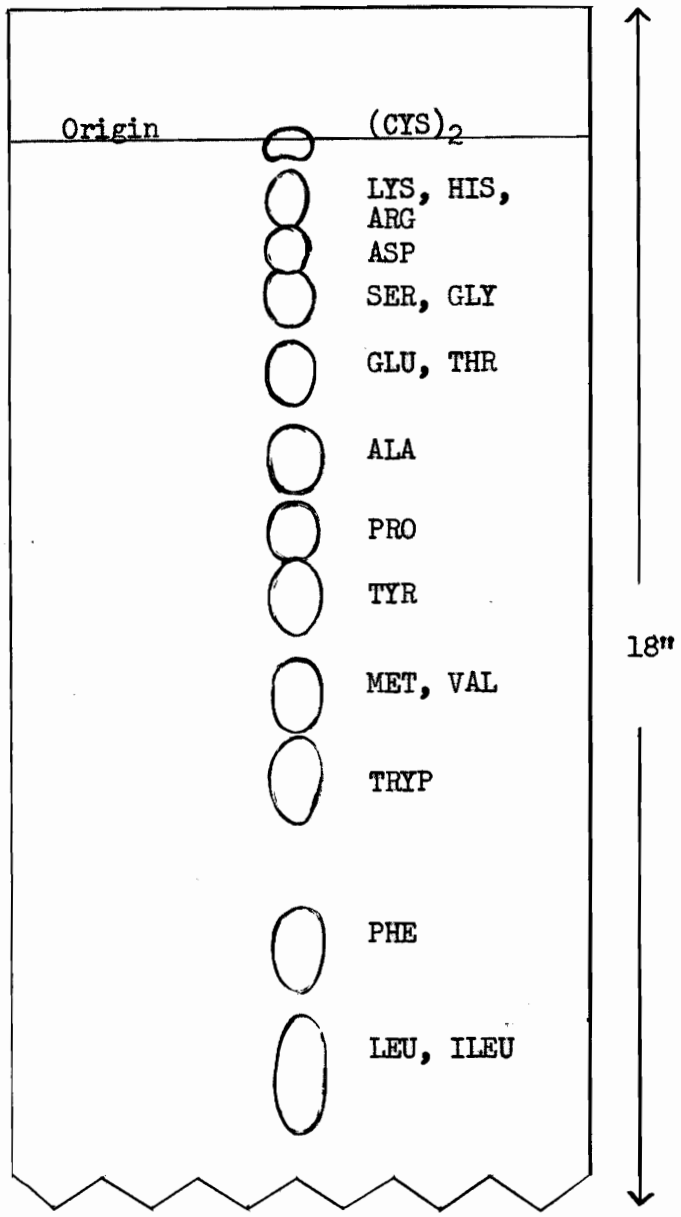

Figure 5. The Approximate Position of Amino Acids on Whatman No. 1 Paper After Development With Butanol-Acetic Acid for 40 Hours.

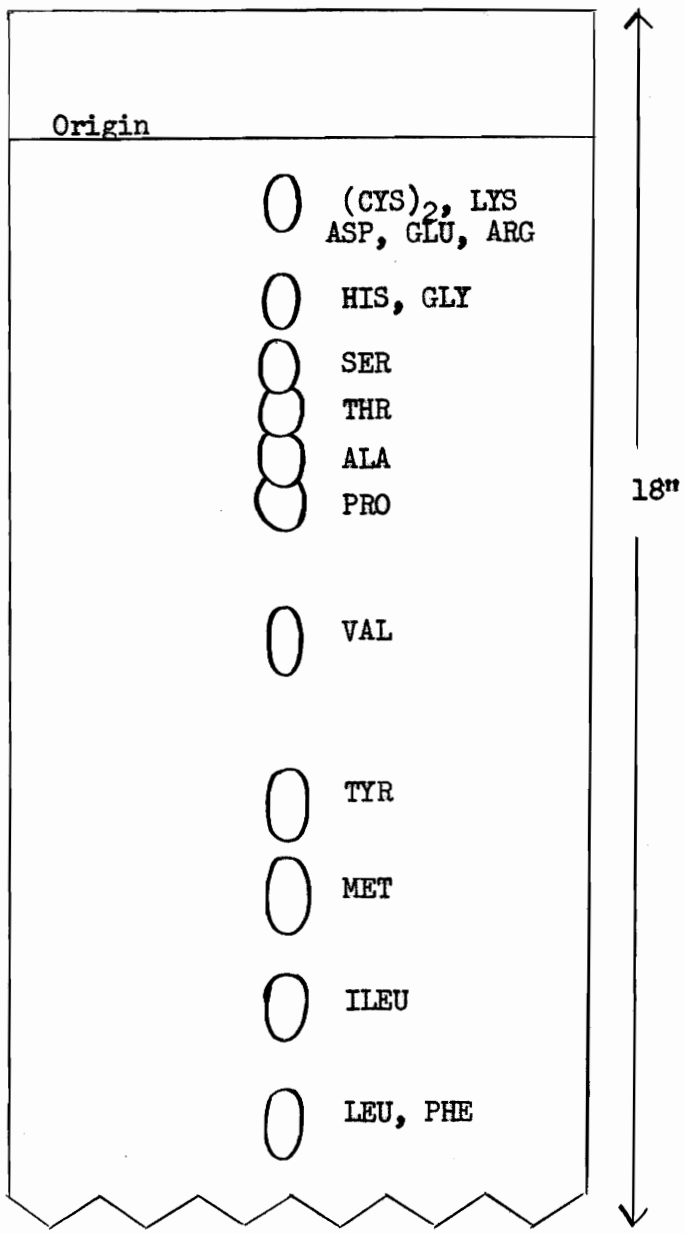

Figure 6. The Approximate Position of Amino Acids on Whatman No. 1 Paper After Development With Tertiary Amyl Alcohol for 4 Days.

paper for 40 hours with butanol-acetic acid.

b. Tertiary amyl alcohol. Work (116) reported that tertiary amyl alcohol saturated with water would effect the separation of leucine and isoleucine, valine and methionine in an atmosphere of diethylamine. Chromatograms on #1 paper (pinked) require 4 to 5 days for the separation of leucine and isoleucine. The separation of amino acids achieved with this system is shown diagramatically in Figure 6.

c. Phenol-water-ammonia. The purification of phenol and the preparation of the best phenol-water mixture for paper chromatography has been described by Draper and Pollard (27). This solvent, in an atmosphere of ammonia, separates serine and glycine, glutamic and threonine, lysine and arginine, pairs that are not separated on butanol-acetic acid chromatograms. Figure 7 shows a typical separation.

d. Ninhydrin reagent. When chromatograms are sprayed with the ninhydrin reagent of Levy and Chung (52), several different colours are evident. The colours of the various amino acids after reaction with this ninhydrin reagent are listed below:

cystine, lysine	-	dark purple
proline	-	yellow
phenylalanine	-	turquoise-grey
aspartic acid	-	turquoise
tryptophan	-	brown
tyrosine, threonine and serine	-	grey-brown
histidine	-	orange-brown
arginine	-	pink-purple
glycine	-	red-purple
others	-	purple

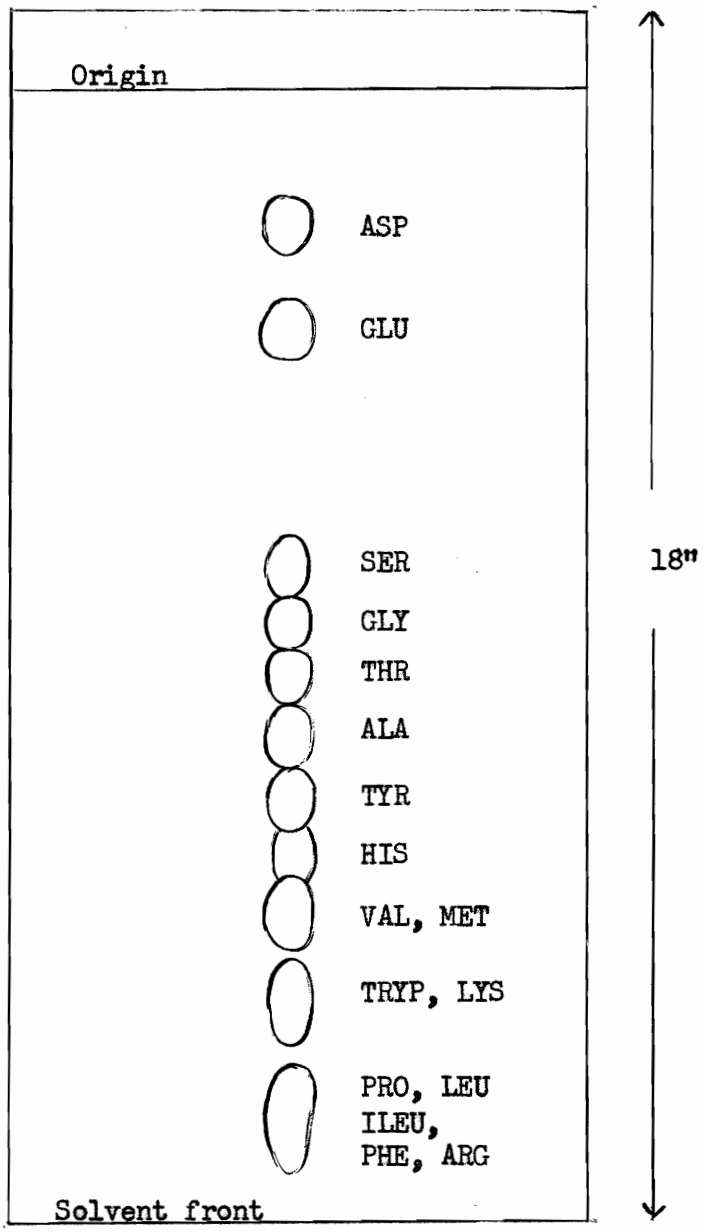

Figure 7. The Approximate Position of Amino Acids on Washed Whatman No. 1 Paper After Development With Phenol-Water-NH_3 for 24 Hours.

The ninhydrin reagent producing these colours is prepared by mixing 50 ml. of 0.1 per cent ninhydrin in 95 per cent ethanol, 2 ml. of technical grade collidine (pure collidine is unsatisfactory) and 15 ml. of glacial acetic acid. The colours fade quickly at very high temperatures and after 24 hours at room temperature.

F. C-Terminal Amino Acids of γ-Globulins

1. Reaction of Protein with Carboxypeptidase.

Grassmann (34) first made use of the enzyme, carboxypeptidase, for the determination of C-terminal groups of peptides. He demonstrated that the C-terminal group of glutathione is glycine. This enzyme has been used by Lens (52), Thompson (167), and Neurath et al. (33,65) to determine C-terminal groups of proteins. The action of carboxypeptidase on rabbit γ-globulin IV, rabbit antibody XIV, human myeloma globulin A and human γ-globulin II-1,2 has been studied by the method of Thompson (107). The liberated amino acids were identified by chromatography on Whatman #1 paper with butanol-acetic acid or phenol-NH_3 as described in Section III-E,6.

2. Control Experiments.

An enzyme control and a protein control were carried out with each experiment. A flask containing carboxypeptidase and buffer was incubated at 37° and aliquots were taken at the same time as, and treated similarly to the complete system. Also, a solution of protein and buffer with no enzyme was checked for spontaneous breakdown of protein.

G. Amino Acid Composition of γ-Globulins

1. Hydrolysis of Proteins.

Smith and Stockell (99) have shown that hydrolysis at 105° with 6 N HCl for 18 to 24 hours did not produce complete breakdown of the protein. Many peptides of valine, leucine and isoleucine were present in the hydrolysates. Also, longer periods of hydrolysis, although effecting hydrolysis of these peptides, caused the destruction of some other amino acids. For this reason, samples of each protein were hydrolyzed for 20 and for 70 hours. The hydrolysis of the protein was performed in sealed tubes of thick Pyrex glass at 105° with approximately 500 volumes of 6 N HCl. The tubes were evacuated to 12 to 15 mm. Hg. before they were sealed to decrease the amount of humin formed. After hydrolysis, the solution was repeatedly concentrated in vacuo at 40 to 50°. The residues were transferred to a volumetric flask, made up to volume with deionized water and preserved with thymol. Solutions represented 4 to 5 mg. of original protein per ml. The actual amount of protein was estimated by duplicate micro-Kjeldahl analyses on aliquots of each hydrolysate taking the nitrogen content of the rabbit antibodies and the myeloma proteins as 16.0 per cent.[6]

An additional sample of each protein was oxidized with performic acid before hydrolysis according to the method of Schram et al. (90). To 50 mg. of protein, in a round bottom flask, there was added 25 ml. of cooled performic acid reagent (1 volume of 30 per cent H_2O_2 and 9 volumes of 87 per cent formic acid were allowed to stand at room temperature for 1 hour

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[6] Analyses of two rabbit γ-globulin preparations by Dr. A. Elek gave an average value of 16.0 per cent nitrogen. The nitrogen content of the multiple myeloma proteins has not yet been determined but has been assumed to be 16.0 per cent. Other human γ-globulin preparations have been reported by Smith, Greene and Bartner (97) to be 15.6 to 16.0 per cent nitrogen.

to produce a maximum concentration of performic acid.) The protein solution, after standing in the refrigerator for 15 to 20 hours, was repeatedly concentrated under reduced pressure at 20°. The residue was then refluxed for 20 hours with 50 ml. of 6 N HCl. The hydrolysate was taken to dryness *in vacuo* at 40 to 50° and the residue made up to volume with deionized water. The protein-nitrogen was determined on duplicate aliquots by the micro-Kjeldahl method.

2. Chromatography of Hydrolysates.

Chromatography of the hydrolysates was performed on columns of Dowex 50 essentially as described by Moore and Stein (62). A 0.9 x 100 cm. column was used for acidic and neutral amino acids and a 0.9 x 15 cm. column for the basic amino acids and cysteic acid. The 100 cm. column was equipped with a water jacket so the temperature of the column could be controlled.

A sample of hydrolysate was placed on the 100 cm. column which had been previously washed with 100 ml. of 0.2 N NaOH containing BRIJ 35, a polyethylene glycol ether detergent, and 100 ml. of pH 3.42 citrate buffer. All buffers used in this discussion of amino acid chromatography have been described by Moore and Stein (62). Aspartic acid, threonine, serine, glutamic acid, proline, glycine and alanine are eluted with pH 3.42 buffer at 37.5°. The temperature is then raised to 50° and valine, methionine, isoleucine and leucine are eluted with pH 4.25 buffer. Tyrosine and phenylalanine are then eluted from the column with this buffer when the temperature is raised to 75°.

A sample of hydrolysate was placed on the 15 cm. column which had previously been washed as described above and buffered at pH 5.0. When the

neutral and acidic amino acids had been eluted with pH 5.0 buffer at room temperature, histidine and lysine were removed with pH 6.8 phosphate buffer. Ammonia and arginine were released from the column with pH 6.5 citrate buffer.

Aliquots of the oxidized hydrolysates were chromatographed on the 15 cm. column which had been washed with 0.2 N NaOH and buffered to pH 3.42. Cysteic acid was readily eluted with this buffer at room temperature.

The eluates were collected in 1 ml. fractions in a Technicon automatic fraction collector equipped with a drop-counting device. The order in which the various amino acids are eluted from the column has been determined by Smith and Stockell (99) in control experiments with known amino acid mixtures.

3. Quantitative Ninhydrin Method for Analysis of Column Eluates.

The 1 ml. samples of eluate were analyzed by the photometric ninhydrin procedure of Moore and Stein (61). Samples were brought to about pH 5 with appropriate amounts of HCl or NaOH. To each buffered sample, 2.0 ml. of ninhydrin reagent were added and mixing was achieved by shaking. The tubes containing the eluate fractions and the ninhydrin reagent were heated for 30 minutes in a vigorously-boiling water bath. The cooled purple solutions were then diluted with 10 ml. of 1:1 propanol-water. The absorbancies of the solutions were determined at 570 mμ against a distilled water blank using a Coleman junior spectrophotometer, model 6-A. Proline solutions which produce a yellow colour with ninhydrin were read at 440 mμ.

The absorbancy of each tube was plotted against the tube number so that a baseline for each absorbancy peak could be determined. The absorbancy of each tube above the baseline and the sum of the absorbancies of all the tubes

containing a particular amino acid were calculated. The amount of amino acid present was determined by comparison of the total absorbancy with that of the colour produced by standard leucine solutions. All amino acids do not produce the same amount of colour per mole. The individual colour yields from amino acids on a molar basis relative to leucine have been determined by Moore and Stein (61) and checked by Stockell. The amount of each amino acid recovered from the column was expressed as grams of amino acid residue per 100 gm. of protein.

4. Determination of Tryptophan.

Tryptophan is destroyed during acid hydrolysis and, therefore, the tryptophan content of rabbit antibodies I, VII, VIII and XIV and human myeloma globulins A and B was determined independently.

Because of the relative insolubility of the ether dried specific antibodies and myeloma globulins, the tryptophan content was determined by a method described by Spies (104). Samples (5 mg.) of the proteins were dissolved in 10 ml. of p-dimethylaminobenzaldehyde solution or Ehrlich's reagent (300 mg. p-dimethylaminobenzaldehyde in 100 ml. 19 N H_2SO_4). These mixtures were kept in darkness until the protein was completely dissolved. This process usually took 12 to 18 hours. Colour was developed by the addition of 0.1 ml. of 0.04 per cent $NaNO_3$ solution. Maximum colour was achieved after storage for 30 minutes in darkness. The absorbancy of each solution was determined at 600 mμ using a Coleman junior spectrophotometer and compared with the absorbancies of a set of standard tryptophan solutions.

Because it was evident that the colours produced by pure tryptophan differed from those produced by the antibodies and human γ-globulins,

absorption spectra of the colour produced by rabbit antibodies, human γ-globulin II-1,2, β-lactoglobulin and tryptophan were measured. The rabbit and human γ-globulins produced colours with an absorption maximum at 630 mμ; tryptophan and β-lactoglobulin colours had a maximum at 600 mμ as Spies had reported. Figure 8 illustrates the absorption spectra of the two groups of compounds.

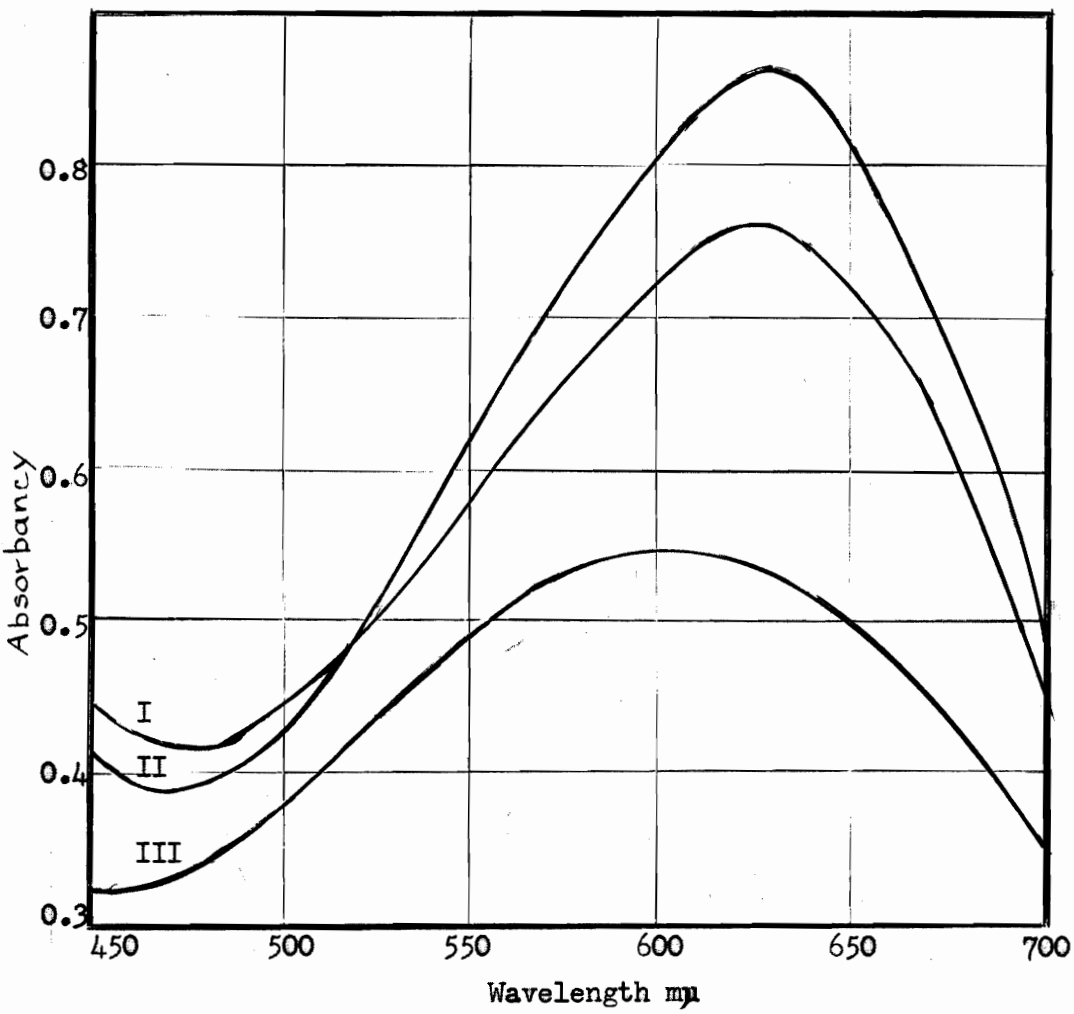


Figure 8. Absorption Spectra of the Colours Produced by Tryptophan and Certain Proteins with Ehrlich's Reagent.

Curve I Rabbit γ-Globulin-VII
Curve II Human γ-Globulin-II-1,2
Curve III L-Tryptophan

It was decided, therefore, to use human γ-globulin II 1,2 as a standard for the estimation of tryptophan in rabbit antibodies and myeloma proteins. The tryptophan content of human γ-globulin II 1,2 was found to be 2.62 $\pm$ .05 gm. of tryptophan per 100 gm. of dry ash free protein by the Sullivan-Hess (105) modification of the Bates method. This value agrees with that reported previously by Smith, Greene and Bartner (97).

5. Determination of Hexose and Hexosamine.

The carbohydrate content of rabbit γ-globulin M was determined by the method of Pirie (78). Glucose was used as standard. The absorbancy of the solutions was determined in a Coleman junior spectrophotometer at 420 mμ.

The hexosamine content of the same protein was determined by the procedure of Palmer, Smyth and Meyer (71). Glucosamine HCl was used as standard. The absorbancy of the solutions was determined in a Coleman junior spectrophotometer at 530 mμ.

IV. RESULTS AND DISCUSSION

A. Physical Studies

1. Electrophoretic Analyses.

The composition and the mobility (μ) of each of the components of the rabbit γ-globulin solutions and the abnormal human globulins are listed in Table 3. Figure 9 is a typical electrophoretic pattern of rabbit γ-globulin. Although some of the rabbit γ-globulin solutions contain as much as 15 per cent albumin or α-globulin it will be evident from later experimental results that these impurities represent a negligible interference. Both components of the two multiple myeloma preparations are γ-globulins. The minor component in both cases has an electrophoretic mobility associated with normal human γ-globulin. Cryoglobulin A, the crude precipitate, however, is seen to contain albumin, α_1-, α_2-, β- and γ-globulin. Cryoglobulin B which was recovered from this electrophoretic run contained the γ-globulin fraction and some β-globulin. The maximum amount of impurity in cryoglobulin B, therefore, cannot exceed 2.6 per cent.

2. Sedimentation Behaviour.

By the definition of Svedberg and Katsurai (106) the sedimentation constant, s, is the sedimentation velocity in unit field. Since the

TABLE 3

ELECTROPHORETIC ANALYSIS OF RABBIT AND

ABNORMAL HUMAN γ-GLOBULINS

All measurements were made in pH 8.5 to 8.6 Veronal buffer at 1.5°.

Preparation	Protein concentration	Mobility, $-\mu \times 10^5$	Gm. component per 100 gm. protein
	per cent	$cm^2\ volt^{-1}\ sec^{-1}$	
A. Rabbit γ-globulins			
I	1.25	1.70	> 99
II	1.25	1.98 6.04	99 1
III	1.03	1.81 5.60	99 1
IV	1.20	1.83 5.65	87 3
V	0.87	1.69 5.55	89 4
VII	0.87	1.80 5.50	87 4
VIII	0.82	1.90 6.20	85 3
XIV	1.03	1.90 5.40	89 4
B. Human γ-globulins			
Cryoglobulin A	0.76	1.5 3.3 4.6 6.0 7.2	76 2 5 3 14
Myeloma A	1.47	0.9 1.8	95 5
Myeloma B	0.87	0.5 1.4	97 3

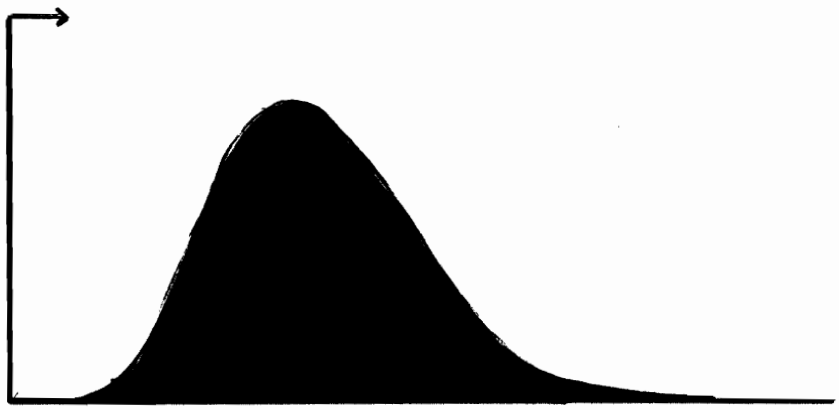


Figure 9. Electrophoretic Pattern of Rabbit γ-Globulin I. This schlieren pattern was photographed after 250 minutes of descending migration at 1.5° in 0.1 M Veronal buffer at pH 8.5. The protein concentration was 1.25 per cent.

sedimentation constant is dependent on the temperature and the medium it is usually converted to a standard value, $s_{20,w}$, corresponding to that obtained in water solution at 20°.

The sedimentation constants in Svedberg Units of the components of each preparation are listed in Table 4. One Svedberg Unit equals 1×10^{-13} cm^2 sec^{-1}. As seen from the table, the sedimentation constant of the main component of each of the eight rabbit γ-globulin solutions is very similar. The average value, 6.31, corresponds to a molecular weight of 158,000. This value has been claculated using the equation:

$$M = \frac{RTs}{D(1 - V\rho)} \qquad (3)$$

A value 3.9×10^{-7}, reported by Kabat (47), has been used for the value of D in this calculation. The calculated molecular weight of 158,000 cannot

be considered as accurate because the sedimentation constant used in the calculation is not necessarily the value at infinite dilution. It does, however, confirm the results reported by Kabat (47). A typical sedimentation pattern for rabbit γ-globulin is reproduced in Figure 10.

Although both myeloma globulins were homogeneous in the electrophoresis, it is evident from Table 4 that preparation A is composed of four components of different molecular weights. This heterogeneity may be due to association of some of the molecules to form dimers, etc. However, it is possible that the main component of multiple myeloma A may be a γ-globulin of higher molecular weight than is normally found in human serum. Putnam (84) has reported the occurrence of abnormally heavy myeloma proteins.

B. Immunological Studies

1. Precipitin Reactions of Rabbit γ-Globulin Solutions with Specific Pneumococcal Polysaccharides.

Heidelberger and Kendall (42) have derived an equation relating the amount of antibody precipitated by a given amount of antigen to the ratio of antibody and antigen at the equivalence point, i.e., the point at which there is neither antigen nor antibody present in the supernatant solution. This equation does not apply in the region of antigen excess.

$$\frac{Ab}{An} = 2R - \frac{R^2}{A} \cdot An \qquad (4)$$

TABLE 4

SEDIMENTATION BEHAVIOUR OF RABBIT AND ABNORMAL HUMAN γ-GLOBULINS.

All preparations were dissolved in a pH 8.5 to 8.6 Veronal buffer with the exception of cryoglobulin A which was dissolved in 0.9 per cent NaCl. Measurements were made at 22 to 24°.

Preparation	Protein concentration	$S_{20,w}$	Gm. component per 100 gm. protein
	per cent	Svedberg u.	
A. Rabbit γ-globulin			
I	1.25	6.32 8.25	92 8
II	1.25	6.29 9.03	93 7
III	1.03	6.30 9.11	92 8
IV	1.20	6.24 9.12	92 8
V	0.87	6.49 9.17	91 9
VII	0.87	6.30 8.81	92 8
VIII	0.82	6.31 8.83	91 9
XIV	1.03	6.25 10.11	93 7
B. Human γ-globulins			
Cryoglobulin A	0.87	23.52 16.13 5.32 4.17	
Myeloma A	0.76	20.0 13.25 8.7 6.6	3 11 78 8
Myeloma B	0.54	6.66	>99

Ab = antibody precipitated

An = antigen added

A = antibody precipitated at the equivalence point

R = Ab/An at the equivalence point

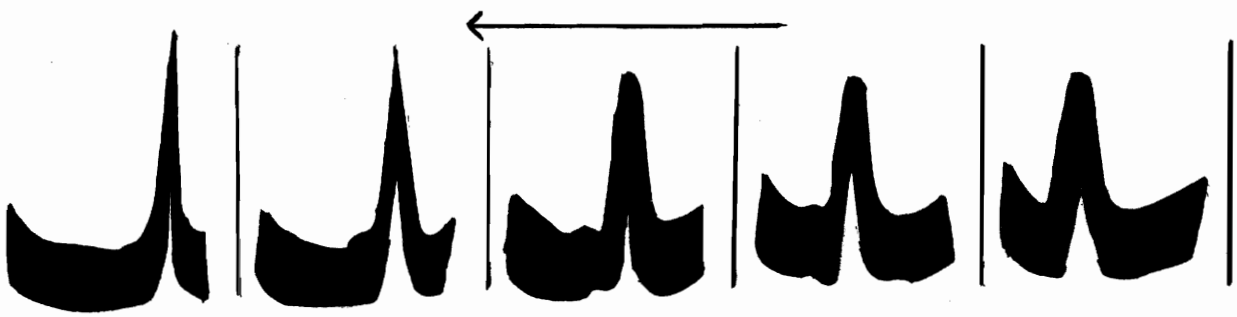


Figure 10. Sedimentation Behaviour of Rabbit γ-Globulin II in the Ultracentrifuge. The arrow indicates the direction of radial migration. This γ-globulin was studied in 0.1 M Veronal buffer (0.1 ionic strength) at pH 8.5. The protein concentration was 1.25 per cent. The picture on the left was taken 20 minutes after the rotor attained full speed (59,780 r.p.m.); subsequent pictures were taken at 16 minute intervals.

According to equation 4, therefore, if the observed values of Ab/An are plotted against An, a straight line should be obtained with a slope equal to R^2/A and an intercept of 2R. From a least squares line drawn through the experimental points, values for the constants in equation 4 can be obtained. From this equation then the amount of Ab precipitated and the composition of the specific precipitate from any mixture of

TABLE 5

PRECIPITIN REACTION OF SI

WITH RABBIT γ-GLOBULIN I

Mg. An(SI) added	Average mg. Ab precipitated	Mg. Ab calculated	$\frac{Ab}{An}$ observed	Test on supernatant	
				excess An	excess Ab
0.025	0.70	0.66	28.0	-	+
0.050	1.12	1.13	22.4	-	+
0.075	1.36	1.40	18.2	-	+
0.100	1.51	1.48	15.1	-	-
0.125	1.36	--	10.9	+	-

Equation: $\frac{Ab}{An} = 30.2 - 153\ An$

TABLE 6

PRECIPITIN REACTION OF SII

WITH RABBIT γ-GLOBULIN II

Mg. An(SII) added	Mg. Ab precipitated	Mg. Ab calculated	$\frac{Ab}{An}$ observed	Test on supernatant	
				excess An	excess Ab
0.25	1.22	1.26	48.8	-	+
0.050	2.30	2.23	46.0	-	+
0.075	2.92	2.90	38.9	-	$\pm$
0.100	2.04	--	20.4	+	-

Equation: $\frac{Ab}{An} = 56 - 240\ An$

TABLE 7

PRECIPITIN REACTION OF SIII

WITH RABBIT γ-GLOBULIN III

Mg. An(SIII) added	Mg. Ab precipitated	Mg. Ab calculated	$\frac{Ab}{An}$ observed	Tests on supernatant	
				excess An	excess Ab
0.025	1.17	1.07	46.6	-	+
0.050	1.65	1.75	33.0	-	+
0.075	1.81	2.06	24.1	-	±
0.100	2.37	2.00	23.7	-	-
0.125	1.93	--	15.4	+	-

Equation: $\frac{Ab}{An} = 50 - 300\ An$

TABLE 8

PRECIPITIN REACTION OF SIV

WITH RABBIT γ_GLOBULIN IV

Mg. An(SIV) added	Mg. Ab precipitated	Mg. Ab calculated	$\frac{Ab}{An}$ observed	Tests on supernatant	
				excess An	excess Ab
0.025	0.98	0.73	39.2	-	+
0.050	1.28	1.27	25.6	-	+
0.075	1.64	1.65	21.9	-	+
0.100	1.83	1.80	18.3	-	±

Equation: $\frac{Ab}{An} = 33.2 - 150\ An$

TABLE 9

PRECIPITIN REACTION OF SV

WITH RABBIT γ-GLOBULIN V

Mg. An(SV) added	Mg. Ab precipi-tated	Mg. Ab calcu-lated	$\frac{Ab}{An}$ observed	Tests on supernatant	
				excess An	excess Ab
0.025	.86	0.83	34.4	-	+
0.0375	1.20	1.12	31.0	-	+
0.050	1.32	1.31	26.4	-	+
0.060	1.41	1.41	23.5	- +	+ -
0.075	1.14	--	15.2	+	-

Equation: $\frac{Ab}{An} = 40 - 275\ An$

TABLE 10

PRECIPITIN REACTION OF SVII

WITH RABBIT γ-GLOBULIN VII

Mg. An(SVII) added	Mg. Ab precipi-tated	Mg. Ab calcu-lated	$\frac{Ab}{An}$ observed	Tests on supernatant	
				excess An	excess Ab
0.05	1.35	1.33	26.9	-	+
0.075	1.49	1.59	19.8	-	+
0.100	1.63	1.60	16.4	-	-
0.125	1.51	--	12.1	+	-

Equation: $\frac{Ab}{An} = 37 - 208\ An$

TABLE 11

PRECIPITIN REACTION OF SVIII

WITH RABBIT γ-GLOBULIN VIII

Mg. An(SVIII) added	Mg. Ab precipitated	Mg. Ab calculated	$\frac{Ab}{An}$ observed	Tests on supernatant	
				excess An	excess Ab
0.025	1.02	1.05	40.8	-	+
0.0375	(1.16)	1.53	(31.0)	-	+
0.050	1.93	1.90	38.6	-	+
0.075	2.59	2.55	34.5	-	+

Equation: $\frac{Ab}{An} = 46 - 159\ An$

TABLE 12

PRECIPITIN REACTION OF SXIV

WITH RABBIT γ-GLOBULIN XIV

Mg. An(SXIV) added	Mg. Ab precipitated	Mg. Ab calculated	$\frac{Ab}{An}$ observed	Tests on supernatant	
				excess An	excess Ab
0.050	1.96	1.64	39.2	-	+
0.075	2.19	2.12	29.3	-	+
0.100	2.37	2.37	23.7	-	+
0.125	2.49	2.38	19.9	+	-

Equation: $\frac{Ab}{An} = 43.2 - 191\ An$

TABLE 13

DATA OBTAINED FROM PRECIPITIN DETERMINATIONS

Type	Gm. γ-globulin per 100 ml. solution	Gm. specific antibody per 100 gm. γ-globulin	R = $\frac{\text{intercept}}{2}$	Slope = $\frac{R^2}{A}$	Composition of the specific precipitate at the equivalence point		
					mg. Ab / A	mg. An	gm. An per 100 gm. precipitate
I	20.7	14.9	15.1	153	1.49	0.098	6.2
II	22.5	28.8	28.0	240	3.24	0.116	3.5
III	19.7	20.2	25.0	300	2.08	0.083	3.8
IV	18.9	19.4	16.5	150	1.83	0.110	5.6
V	18.6	15.4	20.0	275	1.45	0.073	3.3
VII	21.6	14.4	18.5	208	1.65	0.089	5.0
VIII	20.0	30.0	23.0	159	3.00	0.130	4.1
XIV	20.8	23.5	21.6	191	2.44	0.113	4.4

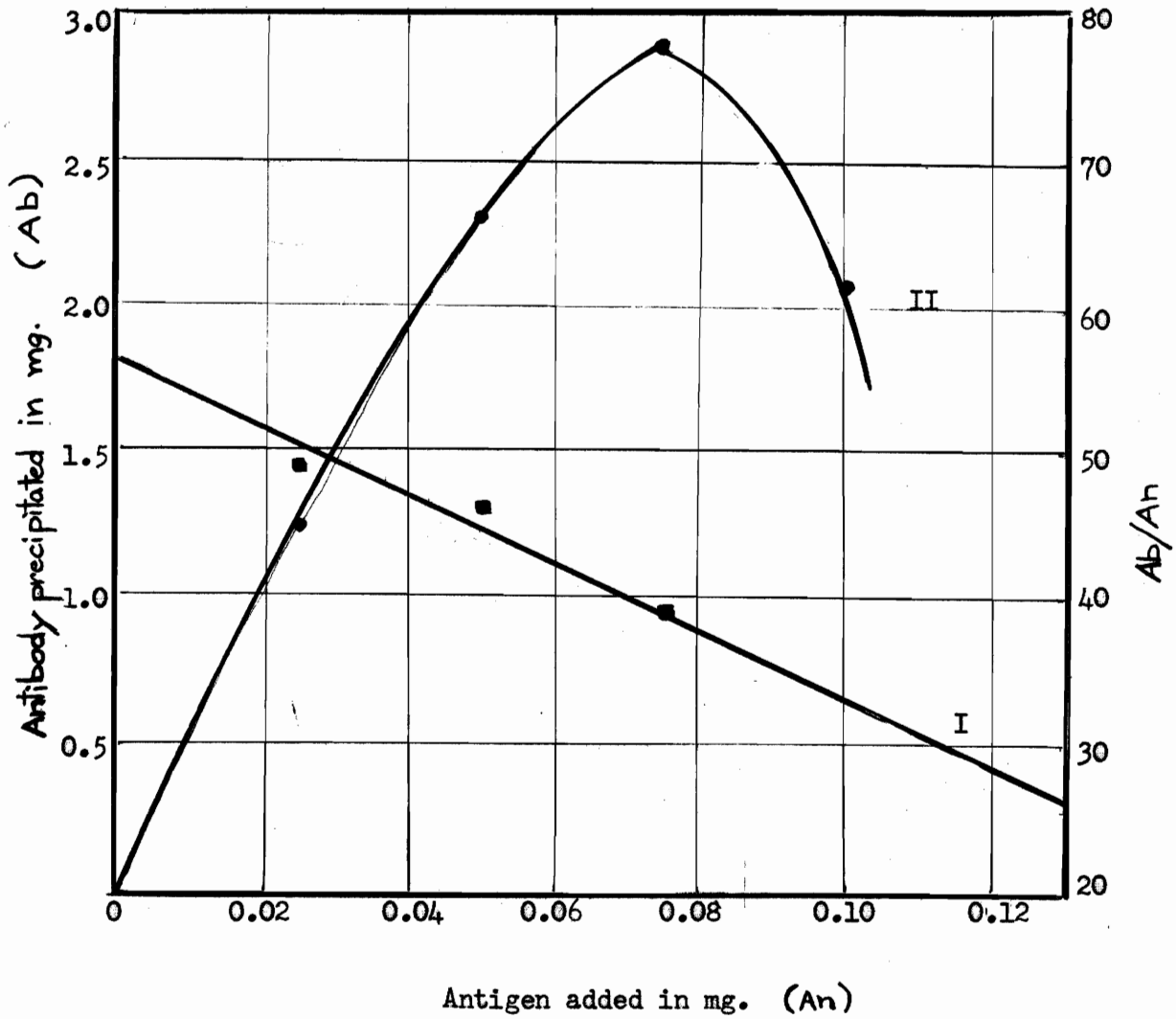


Figure 11. Precipitin Reaction Betwen Pneumococcal Polysaccharide Type II and Rabbit δ-Globulin II.
Curve I is a plot of Ab/An against An added.
Curve II is a plot of Ab precipitated against An added.

antigen and antibody can be calculated. The observed results of the quantitative preciptin determinations on each of the rabbit γ-globulin solutions and the calculated values for the amount of antibody precipitated have been listed in Tables 5 to 12. The pertinent data from these eight tables has been summarized in Table 13.

Figure 11, curve I, is an example of the straight line obtained when Ab/An is plotted against An.

When increasing amounts of a homologous antigen are added to aliquots of an immune serum, increasing amounts of antibody will be precipitated. At one point a maximum amount of antibody will precipitate and tests on the supernatant solution will indicate neither antigen nor antibody. If a greater amount of antigen is added to a similar aliquot of the serum, a decrease in the amount of precipitated antibody will be observed. If, however, the antigen preparation is a mixture of two or more antigens, this decrease will not be apparent. Instead, when the amount of antibody precipitated is plotted against the amount of antigen added, the curve obtained will have a very broad maximum. Curve II, figure 11 represents the characteristic curve for a pure antigen. The data obtained from preciptin reactions with all polysaccharides except SVIII when plotted in this manner produced curves with a maximum. These antigens, therefore, appear to be pure. In the case of SVIII, insufficient antigen was added to reach the equivalence point so that no decision concerning the purity of SVIII can be reached.

2. Specific Antigen-Antibody Precipitates.

The eight rabbit antibodies were precipitated with an amount of specific polysaccharide calculated to bring the mixture to the equivalence point.

The supernatant solutions contained neither excess antigen nor antibody. The composition of these preparations calculated from the equation 4 in Section IV-B,1 is given in Table 13.

C. Characterization of DNP-Proteins

1. Stability.

If the free amino groups of the protein are to be determined by Sanger's DNFB method (82), it is essential that the possibility of hydrolysis of the N-terminal amino acids during the preparation of the DNP-protein not be overlooked. If the bond involving the carboxyl groups of the N-terminal amino acid is especially labile, much of this residue would be present in the mother liquor as the DNP-derivative and would not be quantitatively recovered from hydrolysates of the DNP-protein. Also, Thompson (108) has shown that an N-terminal asparagine can often be distinguished from an N-terminal aspartic acid residue by an examination of the mother liquor.

No hydrolysis of human γ-globulin was evident after treatment with an alcoholic solution of DNFB at pH 8.5 for as long as 16 hours. The supernatant solutions from three DNP-human γ-globulin preparations and from DNP-equine γ-globulin contained no free DNP-amino acids or peptides. Rabbit γ-globulins, however, appear to be less stable. Several DNP-amino acids were present in the supernatant solutions of rabbit γ-globulins I and N after treatment with DNFB for only 3 hours. Additional DNP-amino acids were found to be hydrolyzed from rabbit γ-globulin M which was treated with DNFB for 24 hours. The DNP-amino acids present in the supernatant solutions of DNP-rabbit γ-globulin are listed in Table 14. No DNP-derivative

TABLE 14

AMINO ACIDS HYDROLYZED FROM RABBIT

γ-GLOBULINS DURING TREATMENT WITH DNFB

The "reaction time" of γ-globulin I, N and M was 3, 3 and 24 hours respectively.

DNP-amino acid identified	I	N	M
Asparagine	-	-	+
Aspartic acid	+	+	+
Glutamic "	-	-	+
Alanine	+	+	+
Valine	+	+	+
Leucine or isoleucine	+	+	+
Serine	+	+	+
Threonine	±	-	±
ϵ -Lysine	±	+	±

appeared to be present in an amount greater than 0.05 moles per 160,000 gm. of protein. The significance of the presence of these DNP-amino acids in the mother liquors will be discussed in Section IV-F,1.

2. Composition.

To determine the number of free amino groups per molecule of protein by the DNFB method, the weight of dry protein present in the DNP-prepara-

tions must be known. This information can be calculated if the moisture content of the preparation, the amide content of the dry protein and the air-equilibrated DNP-protein and the antigen content of the antibody-antigen complexes are known. The results of the amide and moisture determinations are listed in Table 15. In cases where there was an insufficient quantity of the preparation to determine moisture and amide content, values for the amount of dry protein in the air-equilibrated DNP-proteins have been assumed. These assumed values (in brackets) together with calculated values are listed in Table 16. All values for antibody preparations are corrected for the presence of antigen using the data in Table 13. Equine antibody III was assumed to be 5 per cent antigen. It has not been definitely established, however, whether the antigen-antibody complexes dissociate during the preparation of the DNP-derivatives. The complexes are insoluble in $NaHCO_3$ solution (γ-globulin is not) and remain so on addition of the alcoholic solution of DNFB. It is, therefore, unlikely that dissociation of the precipitates occurs.

D. Absorption Behaviour of DNP-Amino Acids

The characteristic absorption spectrum of a solution of DNP-L-alanine (20 μ M) in 1 per cent $NaHCO_3$ is reproduced in Figure 11. The absorption curves of solutions of DNP-L-aspartic acid and DNP-DL-glutamic acid in 1 per cent $NaHCO_3$ and ϵ-DNP-L-lysine in 1 N HCl were very similar to Figure 12. All four solutions exhibited maximum absorption at 360 mμ. Table 17 lists the absorbancy indices of the four DNP- amino acids at 350 mμ, 360 mμ and 390 mμ. These data agree with those recently

TABLE 15

THE AMIDE AND MOISTURE CONTENT OF

GLOBULIN PREPARATIONS

Protein	Water in air equilibrated protein	Amide NH_3 in air equilibrated DNP-protein	Amide NH_3 in dry protein
	per cent	per cent	per cent
1. Human γ-globulin			
II-1,2	5.8	1.00	1.34
II-3	6.2	1.01	1.40
myeloma A	5.6	1.00	1.36
myeloma B	6.2	--	--
2. Bovine			
γ-globulin A	6.6	0.972	1.30
3. Equine			
γ-globulin	6.5	--	1.29
T-globulin	6.5	--	1.28
4. Rabbit			
γ-globulin M	6.5	1.01	1.30
antibody I	6.6	--	--
" VII	6.8	--	--
" VIII	6.8	--	--
" XIV	6.5	--	--

TABLE 16

COMPOSITION OF DNP-PROTEINS

The figures in brackets are assumed values. All antibodies have been corrected for the presence of antigen.

Protein	Dry protein in air-equilibrated DNP-protein
	per cent
1. Human γ-globulin	
II-1,2	74.6
II-3	72.2
cryoglobulin	(75)
myeloma A	(73.5)
myeloma B	(73.5)
2. Bovine	
γ-globulin A	74.5
3. Equine	
γ-globulin	(75)
T-globulin	(75)
antibody III	(71.2)
4. Rabbit	
γ-globulin M	77.8
" N	(")
" I	(")
antibody I	(73)
" II	(75)
" III (IIIa)	(75)
" IV	(73)
" V	(75)
" VII	(74)
" VIII	(74.5)
" XIV	(75)

reported by Rao and Sober (85).

The absorbancy of all ϵ-DNP-lysine solutions (1 N HCl) was, therefore, determined at 390 mμ using an absorbancy index of .0109 x 10^6 liters/mole/cm. Thus, if O-DNP-tyrosine were present, no interference would result (Section III-D,4a). The absorbancy of the neutral and acidic DNP-amino acids was always determined at 350 mμ using the values of a listed in Table 17. The wavelength, 350 mμ, had been used before DNP-L-alanine, DNP-L-aspartic acid and DNP-DL-glutamic acid had been obtained in a form sufficiently pure enough to be used for the preparation of standard solutions and was, therefore, used in all subsequent measurements. The absorbancy index of DNP-DL-serine, DNP-L-valine and DNP-L-isoleucine at 350 mu in 1 per cent $NaHCO_3$ were determined from the absorbancy of standard solutions and are also in Table 17.

E. Free Amino Groups of Serum Globulins

The following results have been calculated assuming the molecular weight of human, bovine, equine and rabbit γ-globulins is 160,000. This is probably very close to the true molecular weight of rabbit and human γ-globulin. Bovine and equine γ-globulin, however, appear to have molecular weights nearer 180,000.

It should be noted that the greatest source of error in these determinations is in the application of the correction factors for the recovery of DNP-amino acids after acid hydrolysis. The recovery values used in all calculations were those reported by Porter and Sanger (88) and are listed in Table 2. These values were determined by hydrolyzing

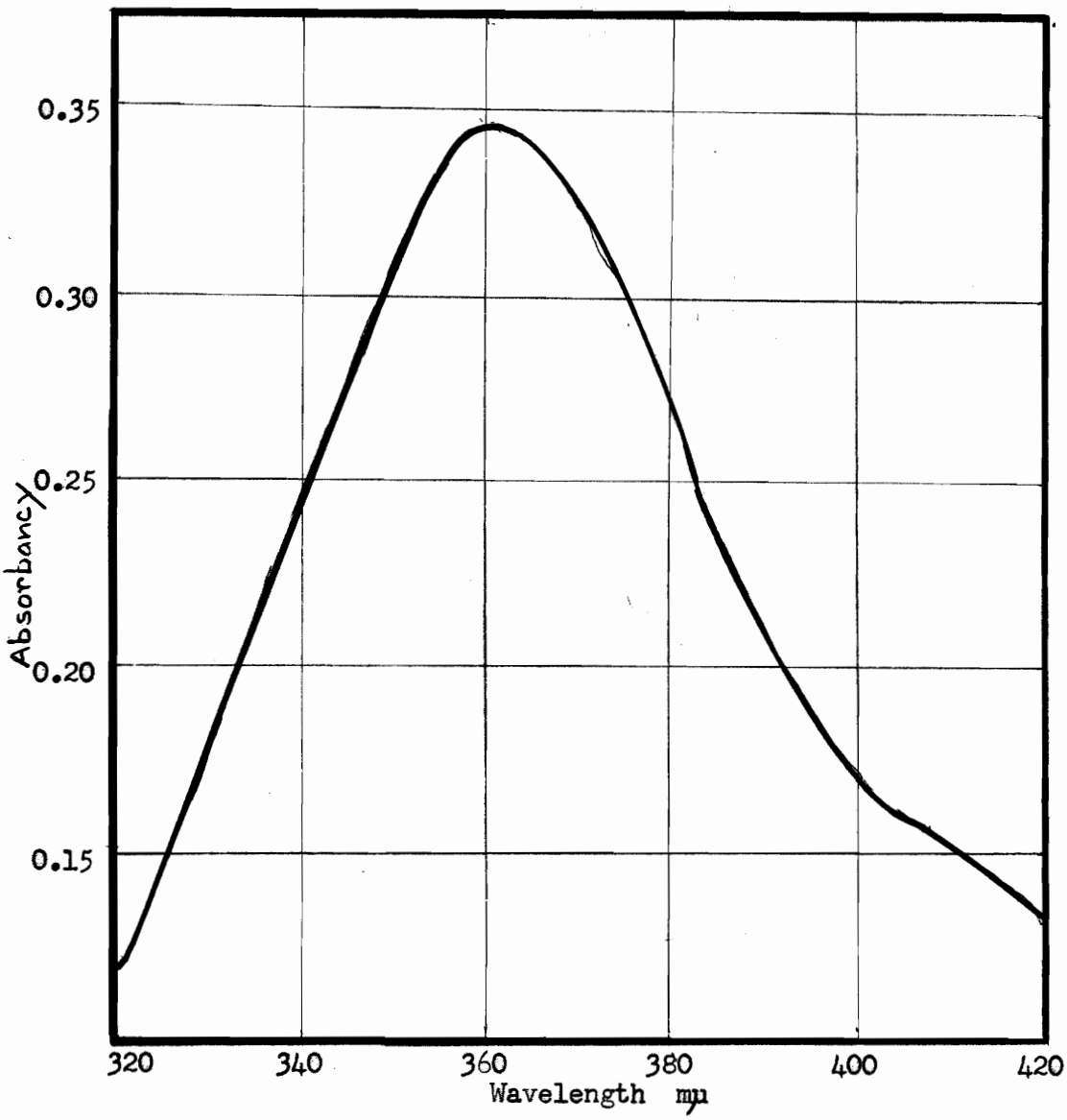


Figure 12. Absorption Spectrum of DNP-L-Alanine. The solution contains 20 μM DNP-L-alanine per liter of 1 per cent $NaHCO_3$.

TABLE 17

ABSORBANCY INDICES OF DNP-AMINO ACIDS

All compounds were dissolved in 1 per cent $NaHCO_3$ solution except ϵ-DNP-L-lysine which was dissolved in 1 N HCl.

DNP-amino acid	a x 10^{-6} liter $mole^{-1}$ cm^{-1}		
	350 mμ	360 mμ	390 mμ
L-Alanine	0.0158	0.0172	0.0103
L-Aspartic acid	0.0161	0.0178	0.0106
DL-Glutamic "	0.0158	0.0176	0.0108
ϵ-L-Lysine	0.0158	0.0180	0.0109
DL-Serine	0.0156	--	--
L-Valine	0.0160	--	--
L-Isoleucine	0.0159	--	--

a known amount of the DNP-amino acid in the presence of a suitable amount of globin.

1. Human γ-Globulins II-1,2 and II-3.

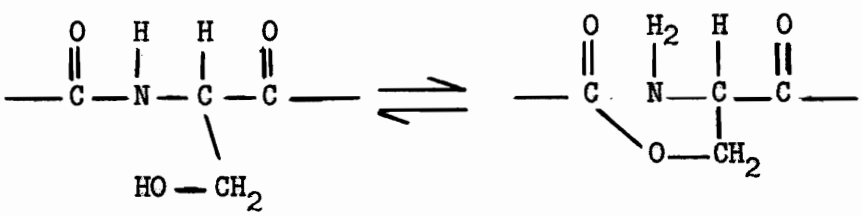
The experimental conditions and the results of several determinations of the free amino groups of human γ-globulins are presented in Tables 18 and 19. γ-Globulin II-1,2 has one N-terminal aspartic acid, approximately two N-terminal glutamic acids, a trace of "N-terminal" serine and 75 lysine residues per mole. γ-Globulin II-3, however, has equal amounts of N-terminal aspartic and glutamic acid, slightly more serine and 73 lysine residues per mole. These results have been confirmed

by Putnam (83).

The lysine content of both γ-globulin fractions agrees well with the microbiological determinations of Smith and Greene (96). The results for human γ-globulins II-1,2B and II-3B (these preparations were shaken with alcoholic DNFB for 16 hours instead of the usual 3) show no significant difference in the recovery of any of the amino acids. Similarly, DNP-human γ-globulin II-3C prepared in pH 6.5 phosphate buffer produced results not differing significantly from the other preparations.

These data confirm the previous indications that normal human γ-globulin is a mixture of several different proteins (Section II-D). Some or all of these different γ-globulin molecules must be composed of either two or more polypeptide chains or branched polypeptide chains otherwise the occurrence of more than one N-terminal residue cannot be explained.

According to Desnuelle and Casal (22) the presence of small amounts of DNP-serine in hydrolysates of DNP-proteins is probably due to lability of the bond involving the amino group of serine. A rearrangement commonly referred to as an "acyl shift" could presumably occur during the isolation of the protein or the preparation of the DNP-derivative. An ester bond could be formed from hydroxyl groups of serine or threonine as follows:



The amino group would then be available for reaction with DNFB.

TABLE 18

FREE AMINO GROUPS OF HUMAN γ-GLOBULIN II-1,2

Mg. of DNP-protein hydrolyzed	Time of hydrolysis in 6 N HCl at 105°	Moles of amino acid per 160,000 gm. of protein				Gm. of lysine per 100 gm. of protein	Gm. of lysine residue per 100 gm. of protein
		aspartic acid	glutamic acid	serine	lysine		
	hours						
120.1	25	(0.71)[a]	2.10	--	73	6.69	5.85
105.4	24	1.07	1.90	--	70	6.43	5.63
206.4	24	1.07	1.80	0.10	--	--	--
212.9	23	1.12	1.70	0.10	77	7.00	6.12
191.7[b]	18	0.98	1.60	0.10	81	7.40	6.49
Average		1.06	1.82	0.10	75	6.88	6.02
Average deviation		±0.04	±0.14		± 4	±0.34	±0.28
Microbiological values for lysine reported by Smith et al. (96)					79	7.2 ± 0.3	

[a] The figures in brackets have been omitted from the average.

[b] This preparation was DNP-human γ-globulin II-1,2B.

TABLE 19

FREE AMINO GROUPS OF HUMAN γ-GLOBULIN II-3

Mg. of DNP-protein hydrolyzed	Hydrolysis time in 6 N HCl at 105°	Moles of amino acid per 160,000 gm. of protein				Gm. constituent per 100 gm. protein	
		aspartic acid	glutamic acid	serine	lysine	lysine	lysine residue
	hours						
138.4	25	(0.64)[a]	0.89	--	77	7.01	6.15
125.0	25	1.07	1.07	--	75	6.83	5.98
121.0	24	(1.40)[a]	(1.30)[a]	--	--	--	--
100.0	24	0.89	1.25	--	71	6.46	5.66
129.1	24	1.20	(1.30)[a]	--	--	--	--
214.2	24	0.98	0.98	0.26	77	7.06	6.20
206.8[b]	24	1.07	0.97	0.24	68	6.18	5.45
205.0[c]	23	0.98	0.98	0.10	--	--	--
183.7[c]	12	0.87	0.94	0.10	72	6.64	5.83
Average		1.01	1.00	0.17	73	6.70	5.88
Average deviation		±0.09	±0.09	±0.08	± 3	±0.27	±0.23
Microbiological values reported by Smith et al.(96)					69	6.3 ± 0.2	

[a] These values have been omitted from the averages.

[b] This preparation was DNP-human γ-globulin II-3C.

[c] These preparations were DNP-human γ-globulin II-3B.

2. Abnormal Human γ-Globulins.

The free amino groups of these γ-globulins are listed in Table 20. Both multiple myeloma globulins possess two N-terminal glutamic acid residues per mole (160,000). A typical chromatogram confirming the presence of DNP-glutamic acid is pictured in Figure 13. The lysine values, however, differ considerably. Multiple myeloma globulin A has the lysine content of normal human γ-globulin whereas, myeloma globulin B has a significantly higher amount. Recently Grisolia and Cohen (35) have isolated myeloma proteins that contained abnormally large amounts of lysine. However, none had such a low mobility as myeloma globulin B ($\mu = 0.5$). Putnam (83) has determined the N-terminal amino acid residues of certain multiple myeloma proteins. Three myeloma proteins with an electrophoretic mobility of -1.1×10^{-5} cm^2/volt/sec were found to have 2 moles of N-terminal aspartic acid. The protein with an electrophoretic mobility of $-0.7 \times 10\text{-}5$ cm^2/ volt/sec had neither aspartic nor glutamic acid as its N-terminal residue.

Cryoglobulin is also an abnormal protein found in the sera of multiple myeloma patients. The results obtained from the pure preparation, cryoglobulin B, suggest that this preparation is a mixture of two or more proteins, one of which has at least three N-terminal residues. Putnam (83) isolated a cryoglobulin yielding 1.8 moles of N-terminal aspartic acid but only 0.14 moles of N-terminal glutamic acid.

3. Rabbit γ-Globulins and Antibodies.

The results in Table 21 demonstrate conclusively that the eight rabbit antipneumococcal antibodies, normal and hyperimmune rabbit γ-globulins, as well as the γ-globulin remaining after antibody has been removed

TABLE 20

FREE AMINO GROUPS OF ABNORMAL HUMAN γ-GLOBULINS

DNP-Protein and amount hydrolyzed	Hydrolysis time in 6 N HCl at 105°	Moles of amino acid per 160,000 gm. protein			Gm. constituent per 100 gm. protein	
		aspartic acid	glutamic acid	lysine	lysine	lysine residue
	hours					
Cryoglobulin A (86.6 mg.)	16	(1.15)[a]	(1.08)[a]	73	6.62	5.81
Cryoglobulin A (30.0 mg.)	17	1.35	1.25	66	6.01	5.26
Cryoglobulin A (23.0 mg.)	18	1.45	1.26	(59)[a]	(5.42)[a]	(4.74)[a]
Average Cryoglobulin A		1.40	1.26	70	6.32	5.54
Cryoglobulin B (15.5 mg.)	18	1.49	1.53	69	6.26	5.49
Myeloma A (105.8 mg.)	18	--	2.01	70	6.42	5.62
Myeloma B (96.5 mg.)	18	--	2.10	99	9.05	8.00

[a] These values are omitted from the averages.

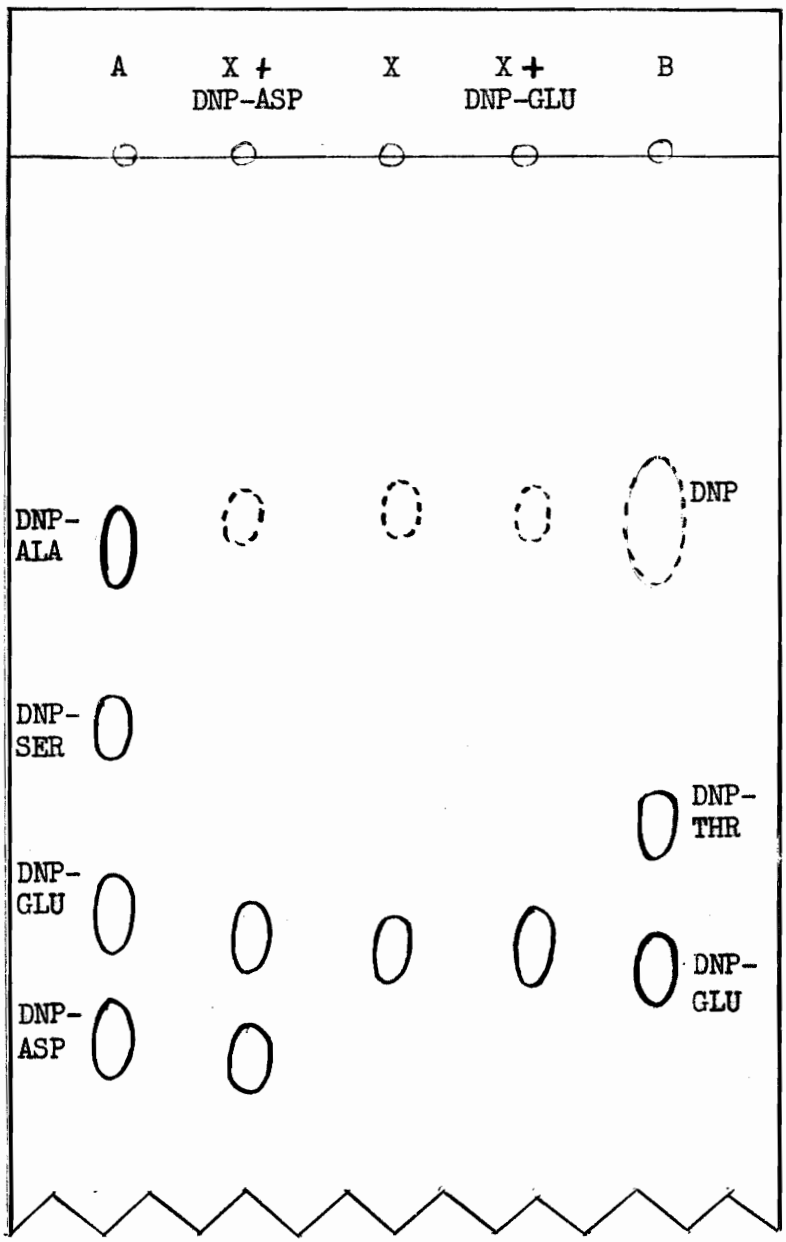


Figure 13. A Chromatogram of the Acidic DNP-Derivative Isolated from an 18 Hour Hydrolysate of DNP-Human Myeloma Globulin A. This chromatogram, on Whatman #1 paper was developed for 22 hours with 1.5 M phosphate buffer. A and B were control mixtures of the DNP-amino acids indicated. X was the DNP-derivative isolated from the hydrolysate and tentatively identified by its behaviour on Celite columns as DNP-glutamic. The four DNP (dinitrophenol) spots were decolourized by HCl fumes.

TABLE 21

FREE AMINO GROUPS OF RABBIT γ-GLOBULINS

Preparation	Moles per 160,000 gm. of protein			Gm. of lysine per 100 gm. of protein	Gm. of lysine residues per 100 gm. of protein
	alanine	aspartic acid	lysine		
γ-Globulin I	1.17	present	72	6.61	5.79
" I	1.18	0.46	77	7.05	6.20
" Ib	0.99	0.41	66	6.02	5.30
" Ib	1.04	0.48	69	6.30	5.52
" M	0.87	0.36	70	6.36	5.56
" M	1.30	0.41	69	6.30	5.52
" N	1.04	0.29	73	6.65	5.87
" N	0.93	0.28	68	6.20	5.40
Antibody I	0.99	0.41	68	6.24	5.47
" II	0.96	0.47	73	6.66	5.82
" II	0.95	0.38	76	6.82	5.95
" III	0.96	0.36	68	6.18	5.40
" IIIa	1.14	0.52	70	6.36	5.56
" IV	1.08	0.48	68	6.17	5.40
" V	0.87	0.35	72	6.58	5.77
" V	0.90	0.50	69	6.25	5.50
" VII	0.89	0.42	68	6.20	5.42
" VIII	1.17	0.36	67	6.08	5.35
" XIV	1.04	0.33	70	6.35	5.56
Average	1.03	0.40	70	6.39	5.60
Average deviation	±0.10	±0.06	± 2	±0.22	±0.19

by specific precipitation all possess the same N-terminal amino acid, alanine, and the same content of lysine, 6.39 per cent. These results confirm the findings of Porter (80). Besides DNP-alanine and ϵ-DNP-lysine, trace amounts of DNP-valine, DNP-serine, DNP-threonine, DNP-leucine and DNP-glutamic acid, as well as significant amounts of DNP-aspartic acid, were found in the hydrolysates of the DNP-proteins. No DNP-glycine, DNP-proline, DNP-arginine or di-DNP-histidine was identified. A chromatogram confirming the identity of DNP-alanine is shown in Figure 14. A possible explanation for the presence of the DNP-derivatives of serine and threonine has previously been discussed. Because it is known that some breakdown of the protein occurs during the preparation of the DNP-protein, the presence of small amounts of other DNP-amino acids in the hydrolysate is not surprising. The presence of relatively large amounts of DNP-aspartic acid, however, is quite unexpected. Free aspartic acid was recovered from the DNP- compound (Section III-D-3c) and identified on a phenol chromatogram by its position and characteristic turquoise color so there can be no question of incorrect identification. Also, no significantly greater amount of DNP- aspartic acid was found in the hydrolysate of rabbit antibody IIIa. Therefore, the presence of aspartic acid cannot be attributed to an adsorption phenomenon. It seems probable that in the rabbit γ-globulin molecule, a sequence such as seryl-aspartyl or threonylaspartyl occurs. Such bonds would be extremely labile and possibly are broken during the treatment with DNFB. The α-amino group of the aspartyl residue could then react with DNFB and DNP-aspartic acid would occur in the hydrolysates. The possibility that some rabbit

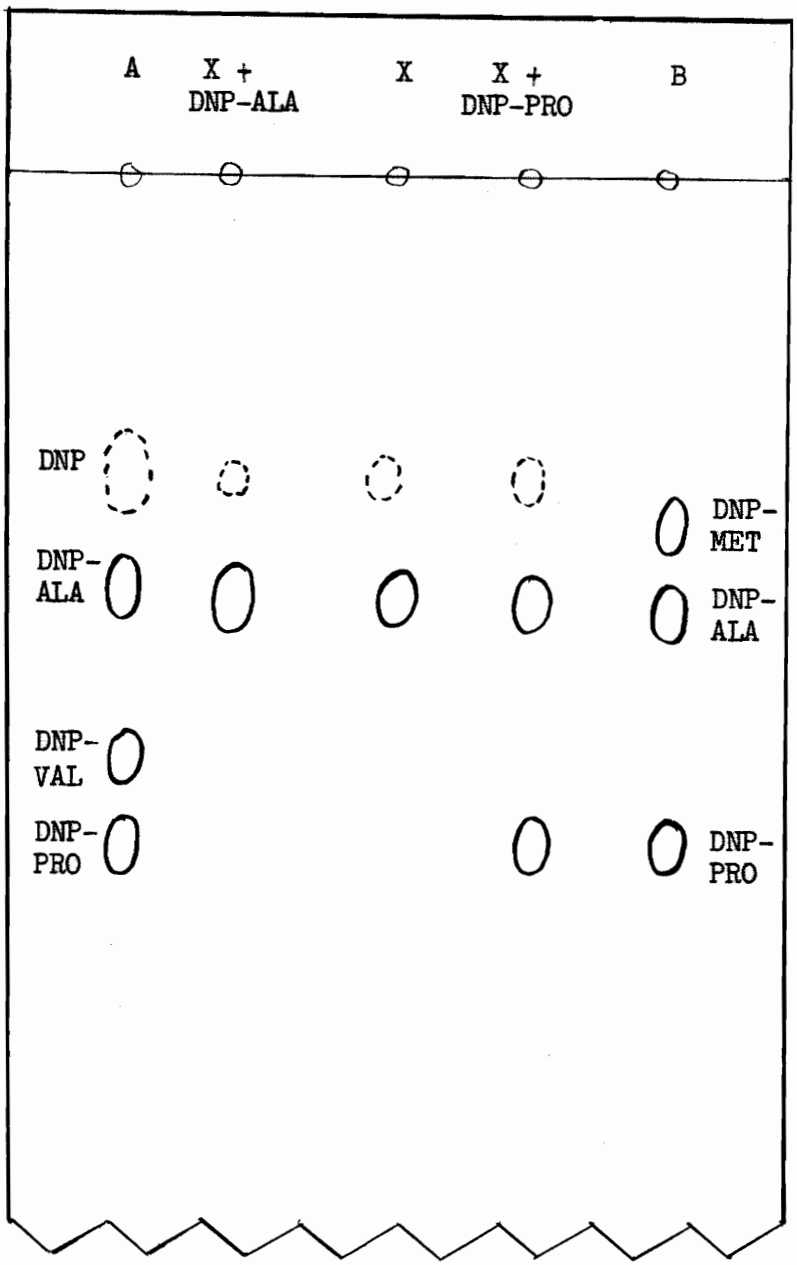


Figure 14. A Chromatogram of a DNP-Derivative Isolated from an 18 Hour Hydrolysate of DNP-Rabbit Antibody VII. This chromatogram on Whatman #1 paper was developed for 25 hours on 1.5 M phosphate buffer. A and B were control mixtures of the DNP-amino acids indicated. X was the DNP-derivative isolated from the hydrolysate and tentatively identified by its behaviour on Celite columns as DNP-alanine or DNP-proline. The DNP spots were decolourized by HCl fumes.

γ-globulin molecules (approximately 40 per cent) may be branched polypeptide chains with one N-terminal aspartic acid and one N-terminal alanine cannot be excluded. Porter (80) did not observe any DNP-aspartic acid in the hydrolysates of DNP-rabbit γ-globulin or antiovalbumin. He employed unbuffered silica-gel columns and mixtures of chloroform and butanol for the separation of DNP-derivatives. Under these conditions DNP-aspartic acid would not move down the column. He reports the presence of a stationary band but, unable to identify it as any DNP-amino acid, he concluded that it was an artefact.

The preparation of the DNP- protein in pH 6.5 phosphate buffer (γ-globulin IB) and the treatment of the protein with DNFB for 24 hours (γ-globulin M) had no significant effect on the recovery of any of the DNP-amino acids.

Because of the existence of a free N-terminal amino group, the amino group of N-terminal alanine cannot be involved in a chemical bond with the antigen (assuming that the antigen-antibody precipitates are not dissociated during the preparation of the DNP- derivatives).

4. Bovine and Equine Globulins.

Rabbit γ-globulin has been found to possess one N-terminal residue; human γ-globulin fractions, two or more. Bovine and equine globulins present still another picture. As seen in Tables 22 and 23 these preparations contain several N-terminal residues. In addition to the amino acids in Table 22, DNP-bovine γ-globulin A hydrolysates contained traces of DNP-leucine or isoleucine. All are present in less than molar quantities. This means that these preparations that are homogeneous in the

TABLE 22

FREE AMINO GROUPS OF BOVINE γ-GLOBULIN A

Mg. of DNP-protein hydrolyzed	Time of hydrolysis in 6 N HCl at 105°	Moles amino acid per 160,000 gm. of protein						Gm. lysine per 100 gm. protein	Gm. lysine residue per 100 gm. protein
		aspartic acid	glutamic acid	serine	alanine	valine	lysine		
	hours								
127.3	24	0.19	0.21	trace	trace	trace	72	6.62	5.81
213.9	19	(0.32)[a]	0.22	0.06	"	"	75	6.94	6.09
217.7	4	0.10	0.08	--	--	--	--	--	--
234.8	18	0.10	0.14	0.12	0.09	0.13	--	--	--
486.9	7	0.13	0.08	0.10	trace	0.09	--	--	--
Average		0.13	0.15	0.09	0.09	0.11	73	6.78	5.95
Average deviation		±0.03	±0.05	±0.02		±0.02	±2	±0.16	±0.14
Microbiological values for lysine reported by Smith et al. (96)							74	6.8 ±0.2	

[a] Figures in brackets are not included in the average.

TABLE 23

FREE AMINO GROUPS OF EQUINE SERUM GLOBULINS

DNP-protein and amount hydrolyzed	Moles amino acid per 160,000 of protein								Gm. of lysine per 100 gm.	Gm. of lysine residue protein	Total moles N-terminal amino acid per 160,000 gm. protein
	asp	glu	ser	thr	ala	val	leu or ileu	lys			
γ-Globulin (423.7 mg.)	0.15	0.09	0.09	0.03	0.06	0.15	0.17	76	6.96 (8.6 ± 0.2)[a]	5.99	0.74
T-Globulin (501.8 mg.)	0.19	0.07	0.16	0.09	0.14	0.16	0.14	74	6.72 (6.7 ± 0.2)[a]	5.89	0.81
Antibody III (106.3 mg.)	0.23	0.10	0.07	--	0.44	0.18	0.09	73	6.66	5.84	1.11
Antibody III (123.0 mg.)	0.23	0.18	0.14	--	0.38	0.18	0.08	74	6.75	5.94	1.19
Average Antibody III	0.023	0.14	0.11	--	0.41	0.18	0.09	74	6.70	5.89	1.15

[a] Values determined microbiologically by Smith et al. (96).

electrophoresis apparatus and the ultracentrifuge are nevertheless heterogeneous mixtures of proteins. Even the specifically precipitated equine antipneumococcus antibody appears to be a mixture of several proteins. Bovine γ-globulin B (not listed in Table 22) has been examined qualitatively for N-terminal amino acid residues. This preparation is also a heterogeneous mixture which contains the same N-terminal residues as bovine γ-globulin A. The amounts present, however, may differ.

The lysine content of bovine γ-globulin A and that of equine T-globulin agree with the values determined by Smith (96) by microbiological methods. The agreement between the two values for equine γ-globulin, however, is not good. This may be due to an unavailability of some of the ϵ-amino groups of lysine for reaction with DNFB (79).

The fact that the total number of N-terminal residues of bovine γ-globulin A and of equine γ-globulin is much less than one can be explained by the existence in the mixtures of proteins having N-terminal tryptophan, arginine, or histidine residues which were not detected or having no N-terminal amino acid residues.

F. N-Terminal Sequence of γ-Globulins

1. Rabbit Antibodies.

The method of separation, the approximate R values and the identification of the DNP-compounds isolated from the partial hydrolysates of the four rabbit antibodies are summarized in schematic form in Figures 15 to 18. These DNP-amino acids and peptides, together with the approximate yield of each compound, are listed in Table 24. A chromatogram of

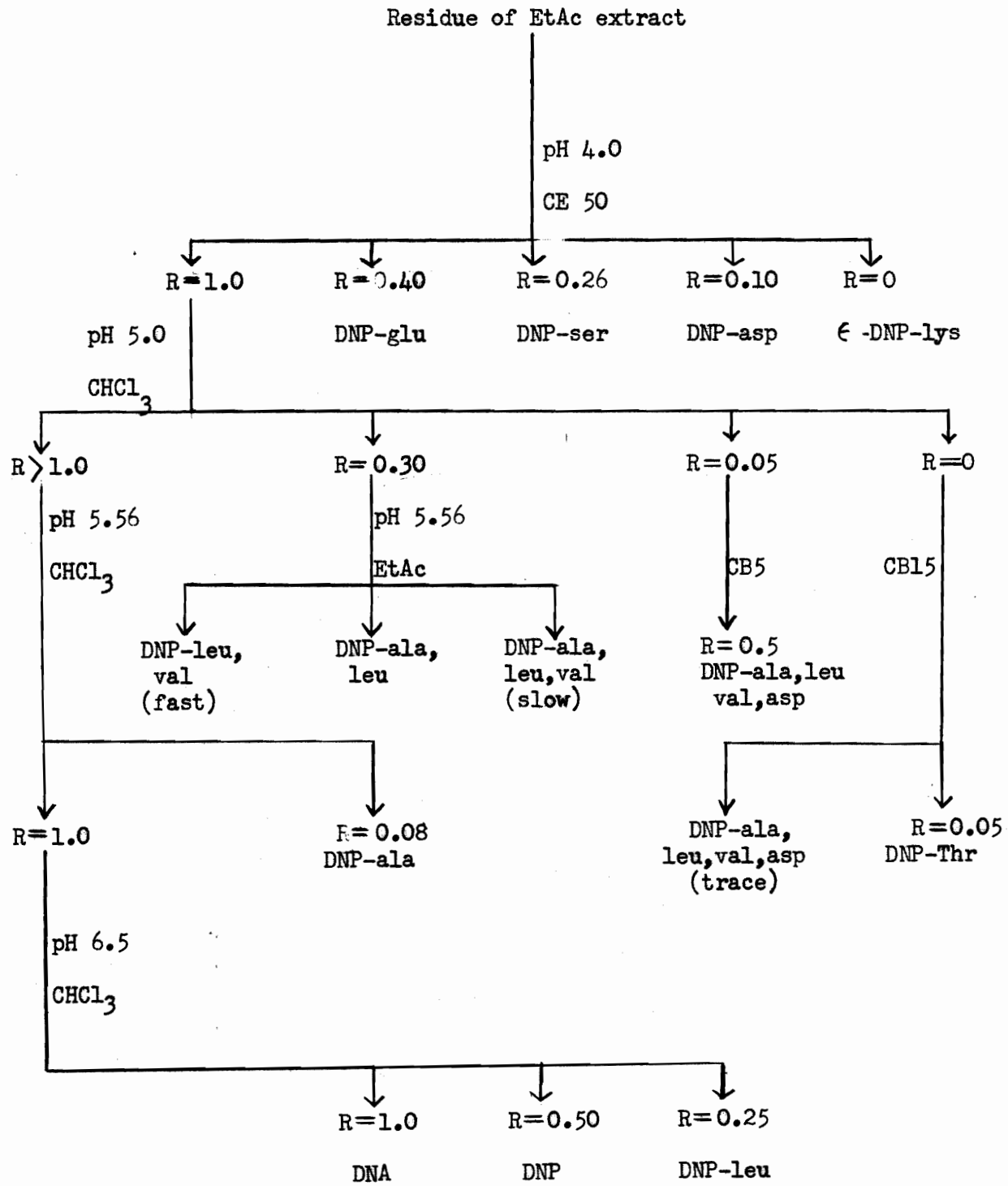


Figure 15. Method of Separation of DNP-Derivatives from a Partial Hydrolysate of Rabbit Antibody I.

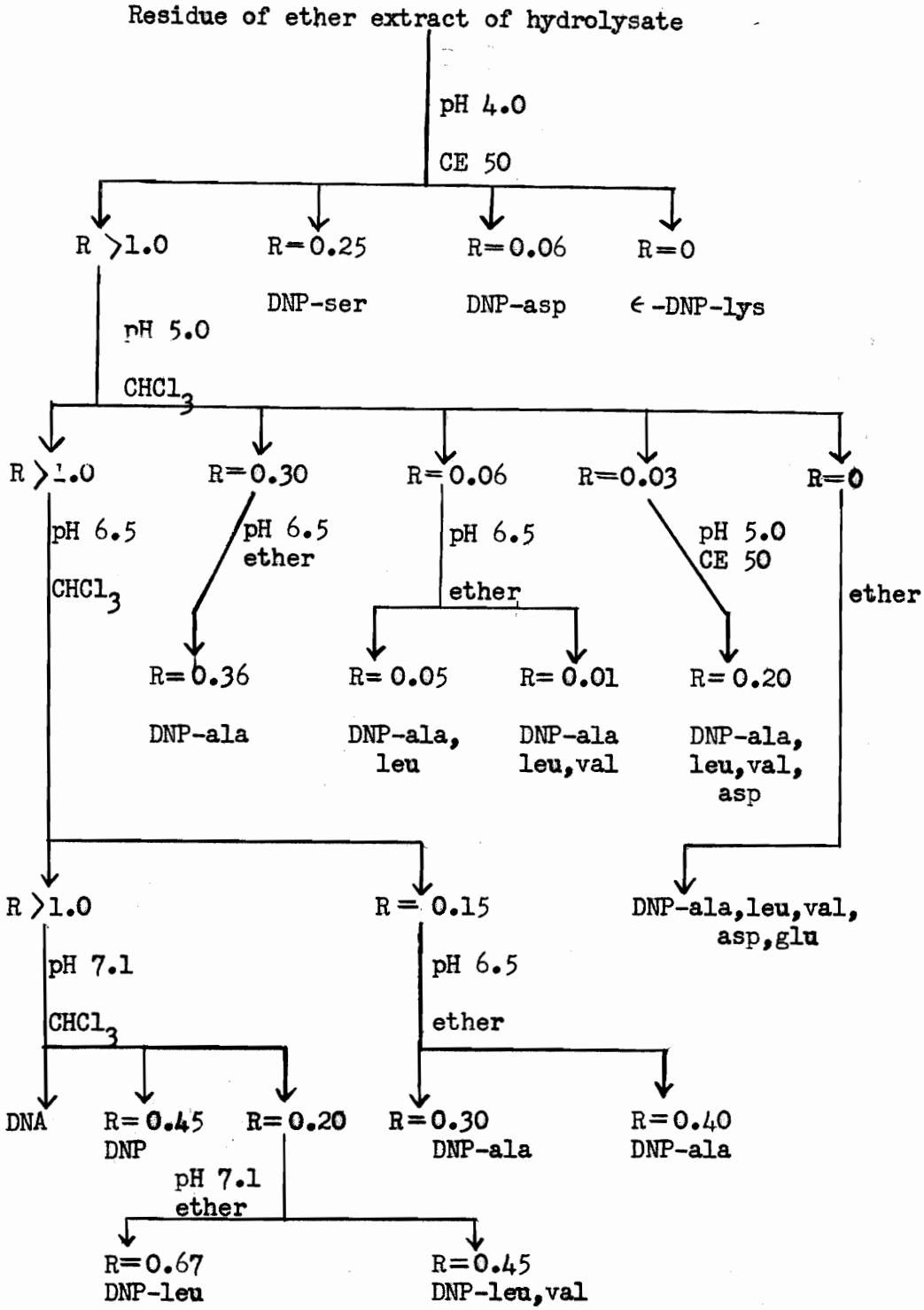


Figure 16. Method of Separation of DNP-Derivatives from a Partial Hydrolysate of Rabbit Antibody VII.

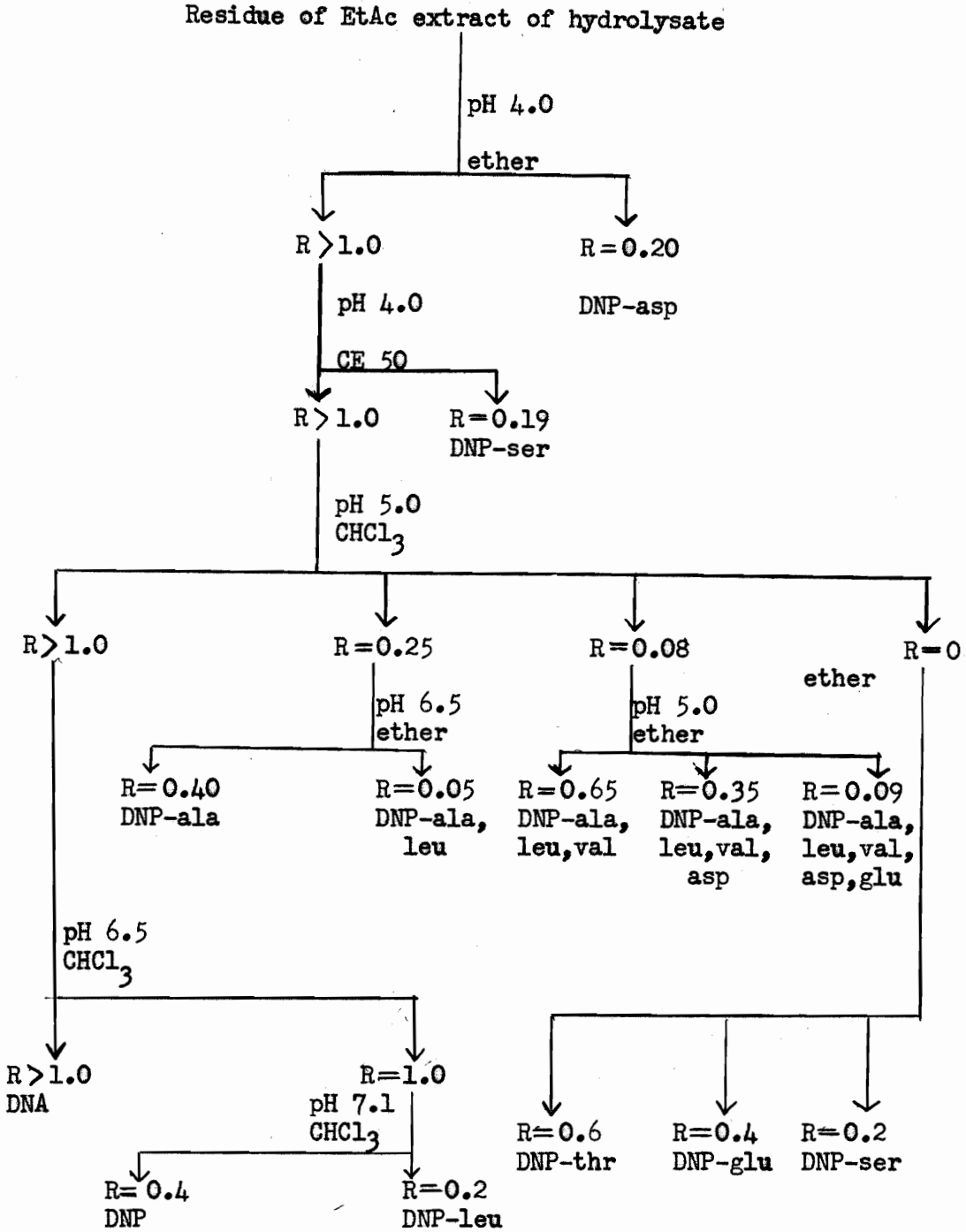


Figure 17. Method of Separation of DNP-Derivatives from a Partial Hydrolysate of DNP-Rabbit Antibody VIII.

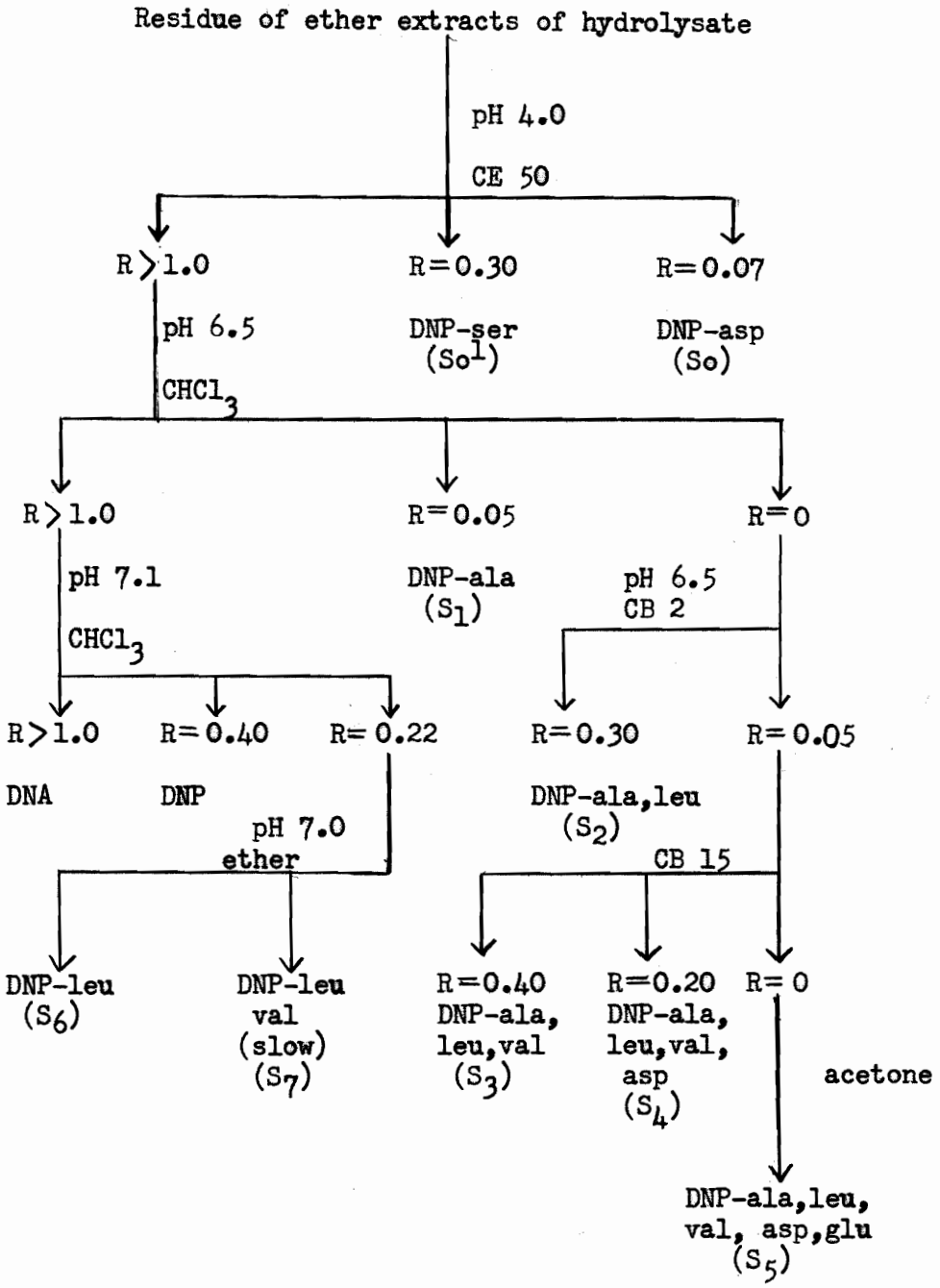


Figure 18. Method of Separation of DNP-Derivatives from a Partial Hydrolysate of Rabbit Antibody XIV. The letters in brackets refer to Figure 19.

TABLE 24

DNP-AMINO ACIDS AND PEPTIDES ISOLATED FROM PARTIAL HYDROLYSATES OF RABBIT ANTIBODIES

Antibody	Hydrolytic conditions	Compounds identified	Yields in moles per 160,000 gm. of protein	Yield of DNP-alanine and its protein
I	6 N HCl, 1 hr. 110°	DNP-aspartic acid	0.25	0.86
		DNP-glutamic acid	trace	
		DNP-threonine	"	
		DNP-serine	0.09	
		DNP-leucine	0.05	
		DNP-leucine (valine)	0.06	
		DNP-alanine	0.33	
		DNP-alanine (leucine)	0.22	
		DNP-alanine (leucine, valine)	0.23	
		DNP-alanine (leucine, valine aspartic acid)	0.08	
VII	12 N HCl, 6 days, 37°	DNP-aspartic acid	0.11	0.73
		DNP-serine	0.03	
		DNP-leucine	0.04	
		DNP-leucine (valine)	0.02	
		DNP-alanine	0.49	
		DNP-alanine (leucine)	0.15	
		DNP-alanine (leucine, valine)	0.04	

TABLE 24 CONT.

Antibody	Hydrolytic conditions	Compounds identified	Yields in moles per 160,000 gm. of protein	Yield of DNP-alanine and its protein
		DNP-alanine (leucine, valine, aspartic acid)	0.03	
		DNP-alanine (leucine, valine, aspartic acid, glutamic acid)	0.02	
VIII	12 N HCl, 6 days, 37°	DNP-aspartic acid	0.20	0.57
		DNP-serine	0.05	
		DNP-glutamic acid	0.05	
		DNP-threonine	0.03	
		DNP-leucine	0.03	
		DNP-alanine	0.33	
		DNP-alanine (leucine)	0.15	
		DNP-alanine (leucine, valine)	0.05	
		DNP-alanine (leucine, valine, aspartic acid)	0.02	
		DNP-alanine (leucine, valine, aspartic acid, glutamic acid)	0.02	
XIV	12 N HCl, 6 days, 37°	DNP-aspartic acid	0.17	0.87
		DNP-serine	0.15	
		DNP-glutamic acid		
		DNP-leucine	0.06	
		DNP-leucine (valine)	0.05	
		DNP-alanine	0.31	

TABLE 24 CONT.

Antibody	Hydrolytic conditions	Compounds identified	Yields in moles per 160,000 gm. or protein	Yield of DNP-alanine and its protein
		DNP-alanine (leucine)	0.15	
		DNP-alanine (leucine, valine)	0.17	
		DNP-alanine (leucine, valine, aspartic acid)	0.17	
		DNP-alanine (leucine, valine, aspartic acid, glutamic acid)	0.07	

the free amino acids isolated from the hydrolysates of the DNP-derivatives found in a partial hydrolysate of DNP-rabbit antibody XIV is pictured in Figure 19. The N-terminal amino acid of each peptide is given in brackets at the top of the chromatogram. These four rabbit antibodies possess the same N-terminal sequence that was found by Porter (80) to occur in normal rabbit γ-globulin and antiovalbumin. The fact that this sequence, alanylleucylvalylaspartylglutamyl, is identical in all the preparations of rabbit γ-globulin studied strongly suggests that the protein-synthesizing cells of the rabbit, unlike those of the human horse and cow, manufacture only one species of γ-globulin.

Porter (80) reported the occurrence of two DNP-peptides which although separable on silica-gel columns, when hydrolyzed, both gave rise to DNP-alanine and free leucine, valine and aspartic acid. He con-

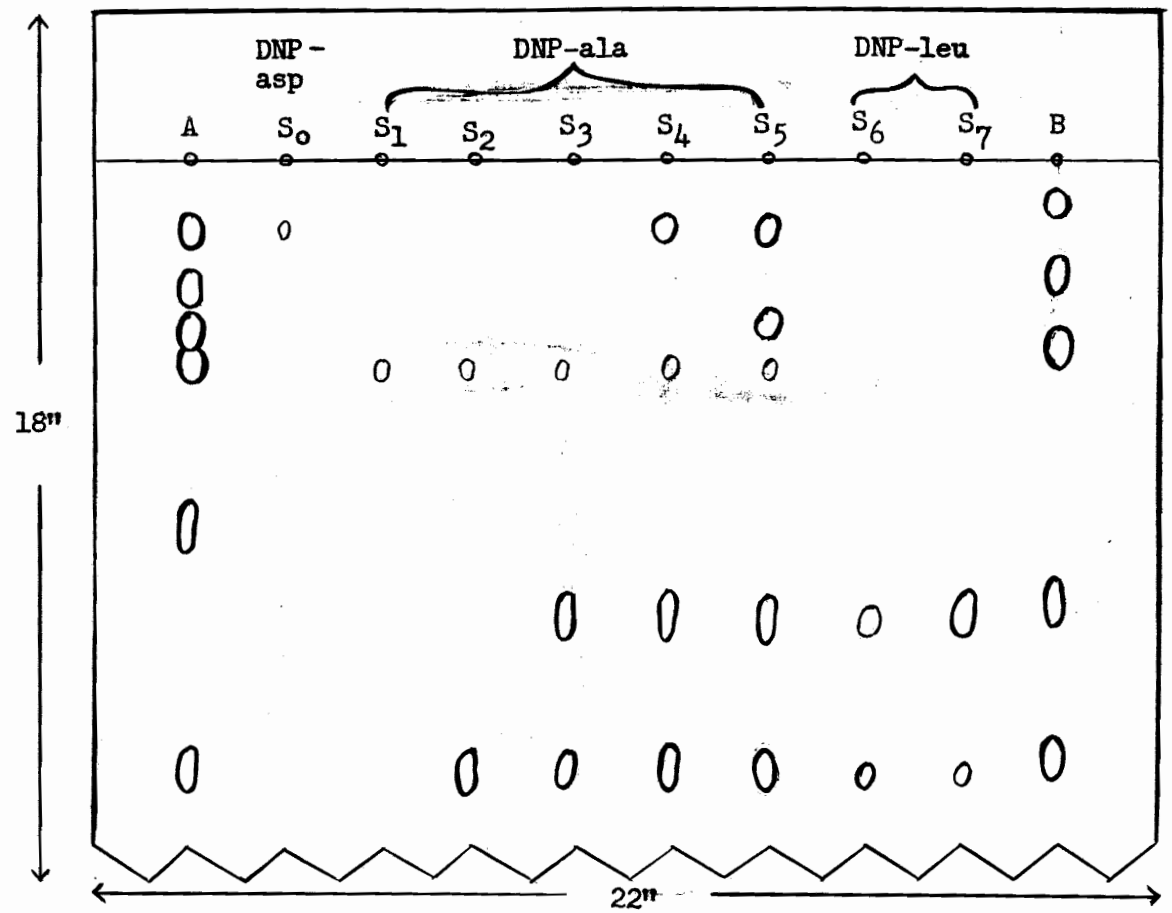


Figure 19. A Chromatogram of the Free Amino Acids Isolated from DNP-Rabbit Antibody XIV. This chromatogram on Whatman #1 paper was developed for 41 hours with butanol-acetic acid. A and B are control mixtures of the amino acids indicated. S_o, S_1 etc. refer to the acid fraction of the hydrolysates of the DNP-derivatives so designated in Figure 18. The DNP-amino acids listed at the top of this chromatogram were found in the ether extracts of the respective hydrolysates.

cluded that the presence of an amide group on the aspartyl residue in one of the peptides explained this observation. From each of the partial hydrolysates of the four antibodies, only one peptide containing alanine, leucine, valine and aspartic acid was isolated. However, because the bands on the column corresponding to this compound were diffuse, it is quite possible that two different peptides were present but not resolved.

No pentapeptide was isolated from the partial hydrolysate of DNP antibody I. This particular hydrolysis was performed in 6 N HCl rather than 12 N HCl, a condition which would presumably facilitate the cleavage of the aspartylglutamyl bond.

Worthy of note is the occurrence of DNP-leucyl valine in three of the four hydrolysates. In the complete hydrolysates of DNP-rabbit antibodies and γ-globulins small amounts of DNP- leucine and DNP- valine were present (Section IV-E,3). This fact, together with the occurrence of small amounts of DNP-leucylvaline suggest that rabbit γ-globulins exist which lack the N-terminal alanine or alanylleucine residues. These incomplete proteins also appear to be precipitated by the various antigens indicating that N-terminal alanine, for example, is not necessary for antibody activity. Porter (80), however, has isolated large polypeptides from papain digests of rabbit antiovalbumin which are specific inhibitors of the antigen-antibody reaction and which possess the N-terminal sequence, ala. leu. val. asp. These incomplete proteins may be artefacts formed during treatment with DNFB. As seen in Table 14, the DNP-amino acids found in the mother liquor of the DNP-protein are primarily those at the

N-terminal end of the chain. Also, the fact that DNP-asparagine is found here substantiates Porter's theory that the aspartic acid in the N-terminal peptide is amidated.

2. Human Multiple Myeloma Globulins.

The method of separation, the approximate R values and the identification of the components in the partial hydrolysates of these two DNP-protines are shown in Figures 20 and 21. It appears that a hydrolysis time of 5 days for multiple myeloma globulin A and 4 days for multiple myeloma globulin B is too great. Although very small amounts of DNP-glutamyl peptides were recovered, the main product of both hydrolyses was DNP-glutamic acid. The amount of each peptide recovered was so small that positive identification of the amino acids was impossible. There is some indication, however, that multiple myeloma globulin A is composed of two different polypeptide chains, one with the N-terminal sequence glutamylalanyl, the other with the sequence glutamylleucyl (or isoleucyl). DNP-glutamylleucine (or isoleucine) was also found in the hydrolysate of multiple myeloma globulin B.

G. C-Terminal Amino Acids of γ-Globulins

1. Rabbit γ-Globulins and Antibodies.

It is evident from the results in Table 25 that the conditions for the determination of C-terminal amino acids with carboxypeptidase have yet to be perfected. The concentration of enzyme used was apparently too great. Sampling at intervals of 15 minutes would have perhaps yielded more conclusive results. The spectrum of amino acids obtained

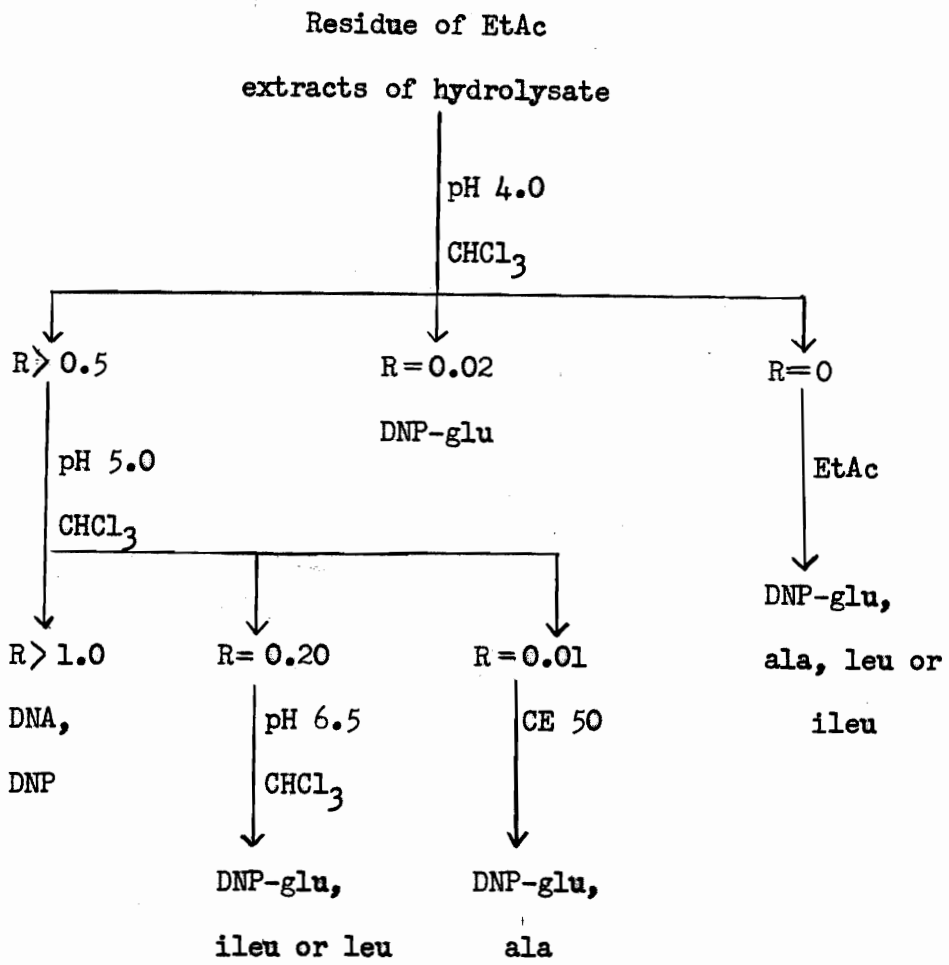


Figure 20. Method of Separation of DNP-Derivatives from a Partial Hydrolysate of DNP-Myeloma Globulin A.

from γ-globulin IV, however, is identical with that obtained from antibody XIV. It is also apparent from these results that the carboxyl group of the C-terminal amino acid of both rabbit γ-globulin and antibody is free. The C-terminal carboxyl group of the antibody, therefore, cannot be involved in a covalent bond with the antigen.

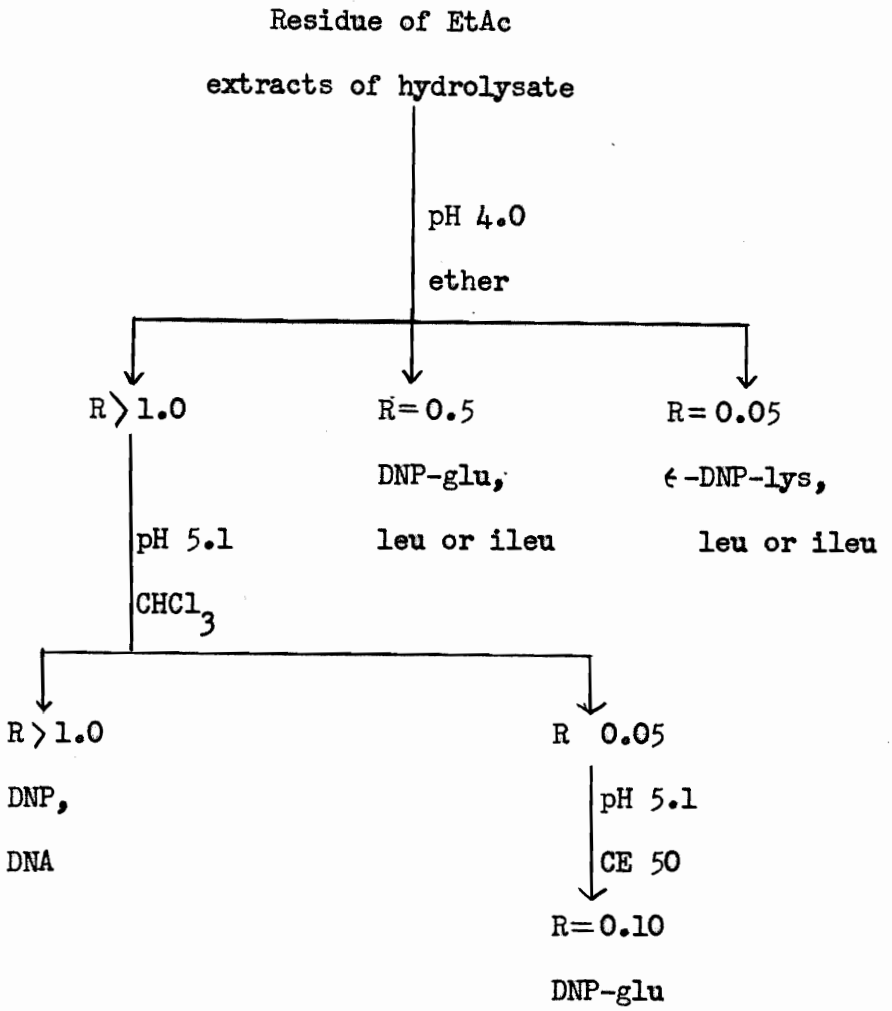


Figure 21. Method of Separation of DNP-Derivatives from a Partial Hydrolysate of DNP-Myeloma Globulin B.

2. Human γ-Globulins.

Even in the few seconds required to take a "zero time" sample of the multiple myeloma globulin A-carboxypeptidase mixture, at least six different amino acids were present in solution. These amino acids were definitely hydrolyzed from the γ-globulin molecule by carboxypeptidase because no amino acids were present in the enzyme or protein control.

TABLE 25

THE ACTION OF CARBOXYPEPTIDASE ON RABBIT γ-GLOBULINS

A. Rabbit γ-globulin IV (160 mg.) and carboxypeptidase (2 mg.) were incubated in 2.5 ml. 0.1 per cent NH_4Ac solution (pH 7.76) at 37°.

Incubation time	Amino acids present in protein free supernatants of 0.4 ml. aliquots (identified on BuOH-HOAc chromatograms)
hours	
0	
1	ala
2	" glu
3	" " asp and or ser (val) leu (and or ileu)
6	" " " " "
23	" " " " " tyr

B. Rabbit γ-globulin IV (200 mg.) and carboxypeptidase (5 mg.) were incubated in 3.5 ml. 0.1 per cent NH_4Ac solution (pH 7.76) at 37°.

Incubation time	Amino acids present in protein free supernatants of 0.4 ml. aliquots (identified on BuOH-HOAc and phenol-NH_3 chromatograms)
hours	
0	
1	ala glu (ser)
3	" " " (asp) val leu ileu (tyr)
5	" " " " " " "
7	" " " " " " "
25	" " " " " " "

TABLE 25 CONT.

C. Rabbit antibody XIV (150 mg.) and carboxypeptidase (5 mg.) were incubated in 3.5 ml. 0.1 per cent NH_4Ac (pH 7.76) at 37°.

Incubation time	Amino acids present in protein free supernatants of 0.5 ml. aliquots (identified on BuOH-HOAc and phenol-NH_3 chromatograms)						
hours							
0							
1	ala	glu	(ser)				
3	"	"	"	(asp)	val	leu (and or ileu)	
5	"	"	"	"	"	"	
7	"	"	"	"	"	"	
25	"	"	"	"	"	"	tyr

An unidentified spot staining pink with ninhydrin-collidine spray was present in 1, 3 and 5 hour samples.

Because of the availability of large amounts of human γ-globulin II-1,2, an attempt was made to establish the proper conditions for the determination of the C-terminal residues of the myeloma proteins. It was found that by incubating 250 mg. of human γ-globulin II-1,2 and 2 mg. of carboxypeptidase in 5.0 ml. of buffer, a slow liberation of amino acids resulted. However, because γ-globulin II-1,2 is a mixture of proteins no other useful information was gained by the experiment. The results are given in Table 26.

TABLE 26

THE ACTION OF CARBOXYPEPTIDASE ON HUMAN γ-GLOBULINS

A. Human multiple myeloma globulin A (250 mg.) and carboxypeptidase (5 mg.) were incubated in 5 ml. 0.1 M NH_4Ac solution (pH 7.5) at 37°.

Incubation time	Amino acids present in protein free supernatants of 0.5 ml. aliquots (identified on BuOH-NOAc and Phenol-NH_3 chromatograms)						
minutes	lys(?)	ser	glu	ala	tyr	met or val	leu or ileu
0	±	++	±	±	++	+	-
10	±	++	±	±	++	++	-
30	±	++	+	+	++	++	±
60	±	++	+	+	++	++	±
100	±	++	++	+	++	++	±
150	±	++	++	++	++	++	+
310	±	++	++	++	+	++	+

B. Human γ-globulin II-1,2 (250 mg.) and carboxypeptidase (2 mg.) were incubated in 5.0 ml. 0.1 M NH_4Ac (pH 7.5) at 37°.

Incubation time	Amino acids present in protein free supernatants of 0.3 ml. aliquots (identified on BuOH-HOAc chromatograms)
hours	
0	
1/2	(serine)
1	serine aspartic or glycine
1 1/2	" "
2	" "
3 1/2	" "
5	" " glutamic or threonine
24	" " "

H. Amino Acid Composition of γ-Globulins

1. Rabbit Antibodies

Figures 22 and 23 are typical examples of the elution curves for the acidic and basic amino acids obtained by chromatography of 70 hour rabbit antibody hydrolysates on Dowex 50 columns. Table 27 presents the results of all the determinations expressed as number of grams per 100 grams of protein of each amino acid residue recovered after chromatography of 20 and 70 hour hydrolysates of the four antibodies.

It is evident from this table that progressive decomposition of serine and threonine occurs with increased hydrolysis time. For this reason, the content of ammonia increases with longer time of hydrolysis. The actual residue per cent of serine, threonine, and ammonia in each protein was obtained by extrapolation to zero time by the method of least squares. This method of extrapolation assumes that the decomposition is zero order. Some investigators, for example, Hirs, have shown that the decomposition of these amino acids in some proteins is actually first order. Up to 70 hours, however, the curves obtained by these two methods do not differ significantly. These four rabbit antibodies contain considerable amounts (4 to 6 per cent) of antigen polysaccharide. This polysaccharide would influence the rate of destruction of serine and threonine considerably. Methionine becomes slowly oxidized during the course of the hydrolysis. Methionine sulfone is the probable oxidation product. No correction for decreased methionine recoveries has been made. Therefore, the average values listed in the tables are presumably low. Methionine sulfone is known to be

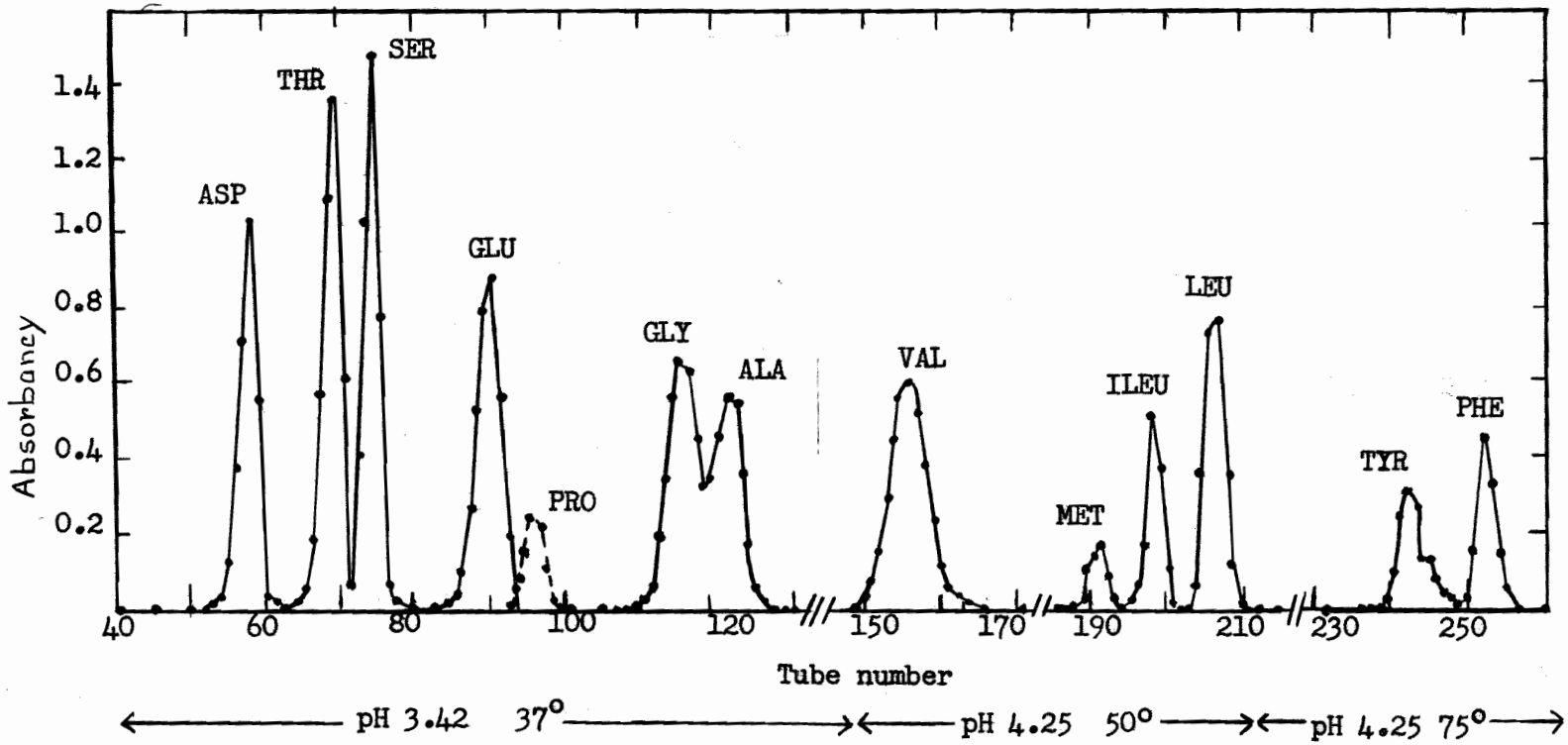


Figure 22. Elution Curves for a 70 Hour Hydrolysate of Rabbit Antibody VII on a 0.9 x 100 cm. Column of Dowex 50.

The ninhydrin colour yields have been corrected for base line colours but not for the different amino acid colour yields. The absorbancy of the proline solutions was determined at 440 mμ: that of the other amino acids at 570 mμ in a Coleman junior spectrophotometer. Protein hydrolysate equivalent to 1.81 mg. of protein was chromatographed in this experiment.

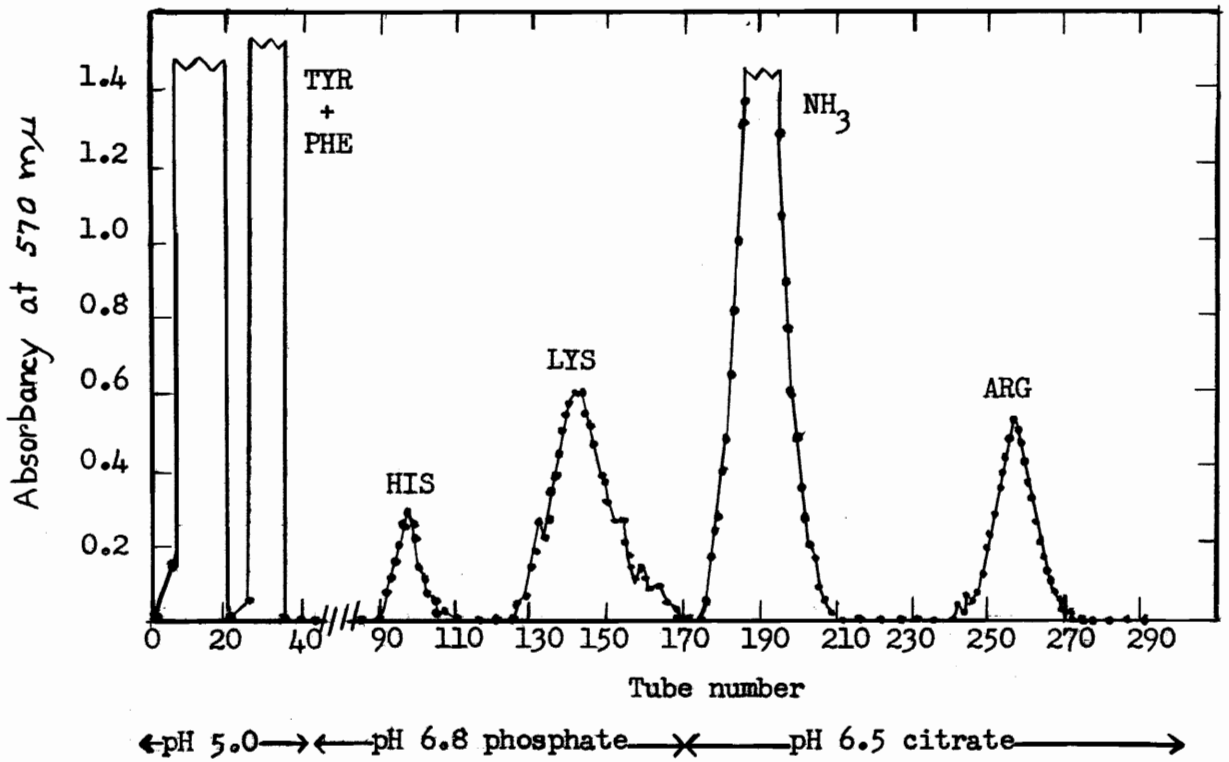


Figure 23. Elution Curves for the Basic Amino Acids and Ammonia for a 70 Hour Hydrolysate of Rabbit Antibody XIV on a 0.9 x 15 cm. Column of Dowex 50. Baseline colour has been subtracted but no correction has been made for the colour yields of the different amino acids. Protein hydrolysate equivalent to 8.80 mg. of protein was chromatographed in the run shown.

TABLE 27

AMINO ACID RECOVERIES FROM RABBIT ANTIBODIES

The data are presented as gm. of amino acid residue per 100 gm. of anhydrous, ash-free protein. The nitrogen content is taken as 16.0 per cent. Values given in parentheses are omitted from the averages. For serine, threonine and ammonia, the values cited in the column as averages were obtained by extrapolation to zero hydrolysis time by the method of least squares.

A. Rabbit Antibody I

Amino acid residue	Time of hydrolysis				Average
	20 hours		70 hours		
Aspartic acid	8.53	8.76	8.40	8.80	8.62 ± 0.13[a]
Threonine	11.44	10.78	9.23	9.26	11.86 ± 0.17
Serine	8.70	7.86	6.50	6.11	9.07 ± 0.31
Glutamic acid	9.77	9.82	10.56	11.00	10.29 ± 0.49
Proline	8.18	6.71	7.09	6.59	7.14 ± 0.52
Glycine	4.79	--	4.26	4.56	4.54 ± 0.18
Alanine	4.78	--	4.43	4.74	4.65 ± 0.15
Valine	(8.01)	--	8.35	8.45	8.40 ± 0.05
Methionine	1.59	--	1.18	1.06	1.28 ± 0.21
Isoleucine	(4.08)	--	4.47	4.02	4.25 ± 0.23
Leucine	7.02	--	7.38	6.85	7.08 ± 0.19
Tyrosine	6.48	--	5.61	6.03	6.04 ± 0.29
Phenylalanine	4.25	--	4.94	4.91	4.70 ± 0.30
Histidine	1.31	1.40	1.28	--	1.33 ± 0.05
Lysine	5.69	5.68	5.59	--	5.65 ± 0.03
Ammonia	(1.81)	1.53	2.40	--	1.21 --
Arginine	4.66	4.48	4.59	--	4.58 ± 0.06
Tryptophan[b]	--	--	--	--	2.47 ± 0.09
1/2 Cystine[c]	--	--	--	--	2.72 ± 0.12
B. Rabbit Antibody VII					
Aspartic acid	7.78	8.13	8.31	8.30	8.13 ± 0.18[a]
Threonine	9.99	10.70	10.03	10.40	10.40 ± 0.27
Serine	8.52	8.61	7.98	7.80	8.84 ± 0.07
Glutamic acid	10.48	10.04	10.12	10.11	10.19 ± 0.15
Proline	7.51	6.56	6.98	7.30	7.09 ± 0.32
Glycine	4.51	4.24	4.28	4.24	4.32 ± 0.10
Alanine	4.77	4.76	4.21	4.55	4.57 ± 0.19
Valine	(7.56)	(7.42)	8.20	8.54	8.37 ± 0.17
Methionine	1.31	1.26	1.35	1.28	1.30 ± 0.03
Isoleucine	(3.19)	(3.31)	3.59	3.77	3.68 ± 0.09
Leucine	6.62	6.31	6.40	6.88	6.55 ± 0.20

TABLE 27 CONT.

Amino acid residue	Time of hydrolysis				Average
	20 hours		70 hours		
Tyrosine	5.67	6.04	5.98	5.97	5.92 ± 0.12
Phenylalanine	4.39	4.51	4.70	4.48	4.52 ± 0.09
Histidine	1.29	1.26	1.16	1.18	1.32 ± 0.02[d]
Lysine	5.70	5.55	5.74	5.87	5.72 ± 0.09
Ammonia	1.43	1.45	1.91	1.95	1.24 ± 0.02
Arginine	(3.91)	4.32	4.37	4.45	4.38 ± 0.05
Tryptophan[b]	--	--	--	--	2.43 ± 0.00
1/2 Cystine[c]	--	--	--	--	2.79 ± 0.19
C. Rabbit Antibody VIII					
Aspartic acid	8.28	8.12	8.51	8.33	8.31 ± 0.11[a]
Threonine	11.22	10.71	9.48	9.58	11.54 ± 0.17
Serine	8.72	8.99	6.70	6.46	9.77 ± 0.13
Glutamic acid	10.20	10.78	10.21	10.70	10.47 ± 0.27
Proline	8.18	7.90	7.31	--	7.80 ± 0.32
Glycine	4.51	--	4.28	4.27	4.35 ± 0.10
Alanine	5.28	--	4.33	4.04	4.55 ± 0.49
Valine	(8.26)	--	9.10	8.91	9.01 ± 0.10
Methionine	1.09	--	1.22	1.25	1.19 ± 0.06
Isoleucine	(3.65)	--	3.66	3.89	3.78 ± 0.12
Leucine	6.85	--	6.66	6.98	6.83 ± 0.10
Tyrosine	6.09	--	5.71	5.69	5.83 ± 0.17
Phenylalanine	(4.33)	--	5.20	5.55	5.38 ± 0.18
Histidine	1.22	1.50	(1.04)	1.35	1.36 ± 0.10
Lysine	5.50	5.90	5.64	5.69	5.68 ± 0.11
Ammonia	(1.75)	1.55	2.11	2.29	1.25 ± 0.09
Arginine	4.41	4.83	4.66	4.88	4.70 ± 0.16
Tryptophan[b]	--	--	--	--	2.43 ± 0.08
1/2 Cystine[c]	--	--	--	--	2.42 ± 0.03
D. Rabbit Antibody XIV					
Aspartic acid	8.30	8.41	8.06	8.68	8.36 ± 8.18[a]
Threonine	10.22	10.35	9.34	9.13	10.71 ± 0.09
Serine	7.78	8.25	6.67	6.79	8.53 ± 0.17
Glutamic acid	10.56	10.61	10.21	10.51	10.47 ± 0.13
Proline	--	6.49	6.95	7.15	6.86 ± 0.25
Glycine	4.37	--	4.09	4.07	4.18 ± 0.13
Alanine	4.04	--	4.35	4.70	4.36 ± 0.22
Valine	(8.36)	--	9.01	8.61	8.81 ± 0.20
Methionine	1.04	--	1.09	1.28	1.14 ± 0.10
Isoleucine	(3.50)	--	3.51	3.38	3.45 ± 0.07

TABLE 27 CONT.

Amino acid residue	Time of hydrolysis				Average
	20 hours		70 hours		
Leucine	6.72	--	6.81	6.90	6.81 ± 0.06
Tyrosine	6.23	--	6.10	5.79	6.04 ± 0.17
Phenylalanine	4.88	--	4.90	5.10	4.96 ± 0.09
Histidine	1.34	1.53	1.28	1.35	1.38 ± 0.08
Lysine	5.31	5.68	5.93	5.50	5.61 ± 0.20
Ammonia	1.55	1.69	2.04	2.19	1.42 ± 0.08
Arginine	4.32	4.43	4.45	4.47	4.42 ± 0.05
Tryptophan[b]	--	--	--	--	2.49 ± 0.01
1/2 Cystine[c]	--	--	--	--	2.69 ± 0.06

[a] Average deviations.
[b] Estimated colorimetrically by the method of Spies (104).
[c] Actual recovery values of cysteic acid calculated as cystine.
[d] Extrapolated value obtained by least squares.

eluted from Dowex 50 columns with threonine. This means that the apparent recovery of threonine especially from 70 hour hydrolysates is high. These errors, however, are small compared with other errors inherent in the method.

Smith and Stockell (99) observed progressive decomposition of serine, threonine, aspartic acid and lysine in hydrolysates of carboxypeptidase and Smith, Stockell and Kimmel (100) reported that decomposition of glutamic acid as well as serine, threonine, lysine and aspartic acid occurred during the hydrolysis of papain. Nevertheless, lysine recoveries from 20 to 70 hour hydrolysates of the rabbit antibodies do not differ.

It is also apparent from these tables that the recovery of valine after 20 hours of hydrolysis is incomplete. For this reason, only the amounts recovered from the 70 hour hydrolysates have been included in

the averages. Sanger (88) has reported that peptide bonds involving the carboxyl group of valine are resistant to hydrolysis. The recovery of isoleucine from 20 hour hydrolysates of rabbit antibody VII was also incomplete. Therefore, the 20 hour values of isoleucine for each of the four antibodies have been omitted from the average.

The values of cystine that appear in these tables are averages of two determinations of cysteic acid that have been calculated as cystine. These values are actual recovery values. It is known, however, that when a weighed amount of pure cystine is oxidized with performic acid, hydrolyzed for 20 hours and chromatographed on Dowex 50 with pH 3.42 buffer, the theoretical amount of cysteic acid is not recovered. The actual amount of cysteic acid recovered appears to be about 80 per cent. Because this factor is not accurately known at present, no correction for low yields of cysteic acid has been made.

The tryptophan content of these four antibodies was determined independently. The values listed in Table 27 are each averages of two determinations.

Table 28 summarizes the combined results of the four determinations on each of the four antibodies. The values for the residue per cent of serine and threonine are extrapolated values of the 16 determinations obtained by the method of least squares. It is questionable whether the overall values for threonine and serine should be obtained in this manner. Because of the presence of antigen polysaccharide, the rate of decomposition of these amino acids may be different with different preparations. For this reason the average of the four extrapolated values is also

TABLE 28

AMINO ACID COMPOSITION OF RABBIT γ-GLOBULIN

The values given are averages for rabbit antibodies I, VII, VIII and XIV

Constituent	Gm. of residue per 100 gm. of protein	Average per cent deviation	Gm. of constituent per 100 gm. of protein	Gm. of nitrogen per 100 gm. of protein	Nitrogen as per cent of total nitrogen
Aspartic acid	8.36	1.7	9.67	1.02	6.38
Threonine	11.22[a]	3.1	13.22	1.55	9.69
Serine	9.05[a]	4.5	10.92	1.46	9.13
Glutamic acid	10.36	1.2	11.80	1.12	7.00
Proline	7.22	4.0	8.56	1.04	6.50
Glycine	4.35	2.3	5.72	1.07	6.69
Alanine	4.53	1.8	5.68	0.89	5.56
Valine	8.65	3.0	10.22	1.22	7.63
Methionine	1.23	4.9	1.40	0.13	0.81
Isoleucine	3.79	6.1	4.39	0.47	2.94
Leucine	6.82	2.1	7.90	0.84	5.25
Tyrosine	5.96	1.3	6.62	0.51	3.19
Phenylalanine	4.89	5.7	5.49	0.47	2.94
Histidine	1.35	1.5	1.53	0.41	2.56
Lysine	5.66	0.7	6.46	1.24	7.75
Ammonia	1.22[b]	0.8	1.30[b]	1.07	6.69
Arginine	4.52	2.7	5.04	1.62	10.13
Tryptophan	2.46	0.8	2.70	0.37	2.31
1/2 Cystine	2.66[c]	4.5	3.13	0.36	2.25
Hexose[d]	0.93	6.5	1.03	—	—
Hexosamine[d]	1.13	1.8	1.26	0.10	0.63
Total	105.14	—	122.74	16.96	106.03

[a] These average values for serine and threonine were obtained by extrapolating by least squares the 16 individual determinations. If instead, the four extrapolated values listed in Table 27 are averaged, the values for gm. of amino acid residue per 100 gm. of protein for threonine and serine are 11.13 and 9.05 respectively. The respective average per cent deviations are 5.1 and 4.1.

[b] These values are omitted from the totals.

[c] This is an actual recovery value.

[d] These constituents were determined on whole rabbit γ-globulin. These are not averages of determinations on the four antibodies.

reported in Table 28. The value used for grams of ammonia residue per 100 grams of protein was determined independently on whole rabbit γ-globulin (Section IV-C,2). Because there is no greater variation between the results for the different antibodies than exists among different determinations of an amino acid on the same antibody, these four antibodies can be considered to have the same amino acid composition. In other words, with the methods of analysis available today, no differences in the composition of these four rabbit antibodies can be detected. However, variations of one or two amino acid residues would not be observed in the hydrolysates of such large protein molecules.

It may not be apparent that the serine and threonine content of these four antibodies does not differ. It has been shown, however, for both these amino acid residues, that the F ratio (the ratio of the variance between the four sets to the variance within each set) is well below the critical value which may be exceeded only once in 20 times if these two variances do not differ. Therefore, the agreement between values for the four antibodies is acceptable as measured by the agreement between determinations within sets.

Rabbit γ-globulin was found to contain hexose and hexosamine. The average values listed in Table 28 are the results of 11 determinations of hexose and 6 determinations of hexosamine on whole rabbit γ-globulin, not antibody. This carbohydrate appears to be the only non-amino acid constituent of rabbit γ-globulin.

It is important to realize that because no pneumococcal polysaccharide-free antibody was available, the carbohydrate content of these four

antibodies has been assumed, not proven, to be identical to that of whole rabbit γ-globulin. This assumption is poor and should be verified.

There are several possible ways that the polysaccharide may be linked to the protein. A hydroxyl or an amino group of the sugar might be joined to the free carboxyl group of an aspartic or glutamic acid residue by an ester or an amide bond. Because carboxypeptidase liberates free amino acids from rabbit γ-globulins, it does not seem probable that the terminal carboxyl group of the protein could be linked to the polysaccharide. Also, because the amino group of N-terminal alanine is available for reaction with DNFB, this amino group cannot form part of an amide bond with the carboxyl group of a hexuronic acid residue in the sugar. It is possible, however, that one or two ε-amino groups of lysine are involved in binding the polysaccharide, although all, or nearly all, of these groups are available for reaction with DNFB. It should be noted that there is no significant difference in the number of lysine residues determined by the DNFB technique of Sanger (86) Table 21, and the number determined by the method of Moore and Stein (62).

As seen in Table 28, the total grams of amino acid residues recovered from 100 grams of protein are 105.14. The nitrogen recovery is also high. An incorrect estimation of the nitrogen content of the protein (16.0 per cent) would influence the weight recovery but would have no effect on the estimated nitrogen recovery, since the quantity of protein in the hydrolysates was estimated from the same nitrogen value. Consistently low Kjeldahl nitrogen determinations on the protein hydrolysates would produce high weight and nitrogen recoveries.

Because the weight of rabbit γ-globulin is completely accounted for by amino acids, ammonia and carbohydrate, the partial specific volume ($\bar{V}$) of this protein can be calculated. The values used for the specific volumes of the amino acid residues are those given by Cohn and Edsall (18). The amide groups have been assigned equally to asparaginyl and glutaminyl residues. This assignment is purely arbitrary. The assignment of all the amide groups to either asparaginyl or glutaminly residues, however, would not change the calculated value for the partial specific volume. The data in Table 29 lead to a value of 0.728 for the partial specific volume of rabbit γ-globulin. Cohn and Edsall (18) report an assumed value of $\bar{V}$ for rabbit antipneumococcus serum globulin of 0.745. This value is based on experimental determinations on closely related proteins.

In Table 30, the number of ionic groups of rabbit γ-globulin is given on the assumption that a single terminal α-carboxyl group is present in the protein. This would be consistent with the single free α-amino group known to occur (Section IV-E,3). The isoelectric point of purified rabbit antibodies to pneumococcus and egg albumin has been reported by Kabat and Mayer (48) to be pH 5.8. Therefore, at the isoelectric point, 11 of the 16 imidazole groups must be uncharged.

The sulphur content of rabbit γ-globulin determined from the content of methionine and cystine (the latter corrected for 82 per cent recovery) is 1.319 grams of sulphur per 100 grams of protein. This value agrees very well with the value 1.39 per cent determined microanalytically by Dr. A. Elek.

TABLE 29

PARTIAL SPECIFIC VOLUME OF RABBIT γ-GLOBULIN

The values of $\bar{V}$ for the amino acid residues are those given by Cohn and Edsall (18)

Constituent	Gm. of residue per 100 gm. of protein (W)	$\bar{V}$	$\bar{V}$ W
Aspartic acid	3.97	0.60	2.38
Asparagine	4.40	0.62	2.73
Threonine	11.22	0.70	7.85
Serine	9.05	0.63	5.70
Glutamic acid	5.42	0.66	3.58
Glutamine	4.94	0.67	3.31
Proline	7.22	0.76	5.49
Glycine	4.35	0.64	2.78
Alanine	4.53	0.74	3.35
Valine	8.65	0.86	7.44
Methionine	1.23	0.75	0.92
Isoleucine	3.79	0.90	3.41
Leucine	6.82	0.90	6.14
Tyrosine	5.96	0.71	4.23
Phenylalanine	4.89	0.77	3.77
Histidine	1.35	0.67	0.90
Lysine	5.66	0.82	4.64
Arginine	4.52	0.70	3.16
Tryptophan	2.46	0.74	1.82
1/2 Cystine	2.66	0.61	1.62
Hexose	0.93	0.65	0.60
Hexosamine	1.13	0.66	0.75
Total	105.14		76.57

$$76.54 \div 105.14 = 0.728 = \bar{V}$$

TABLE 30

IONIC GROUPS OF RABBIT γ-GLOBULIN

The number of ionic groups is calculated on the basis of a molecular weight of 160,000 and on the assumption that there is a single free α-carboxyl group to conform with the single α-amino group.

Acidic residues	Number of moles per 160,000 gm. of protein	Basic residues	Number of moles per 160,000 gm. of protein
Aspartic acid	116	Arginine	46
Glutamic acid	128	Lysine	71
Terminal carboxyl	1	Histidine	16
	245	Terminal α-amino	1
Amide groups	-122		
Total anionic groups	123	Total cationic groups	134

2. Abnormal Human Serum Globulins

a. Myeloma globulin A. Figures 24 and 25 are examples of typical elution chromatograms of a 20 hour hydrolysate of this protein. Table 31 summarizes the recoveries of the amino acids from 20 and 70 hour hydrolysates as well as the extrapolated or average values for each amino acid residue. Because of the progressive decomposition of serine and threonine, the actual values of serine, threonine and ammonia were obtained by the method of least squares. The ammonia content agrees with an independent determination (Table 15). The residue per cent lysine

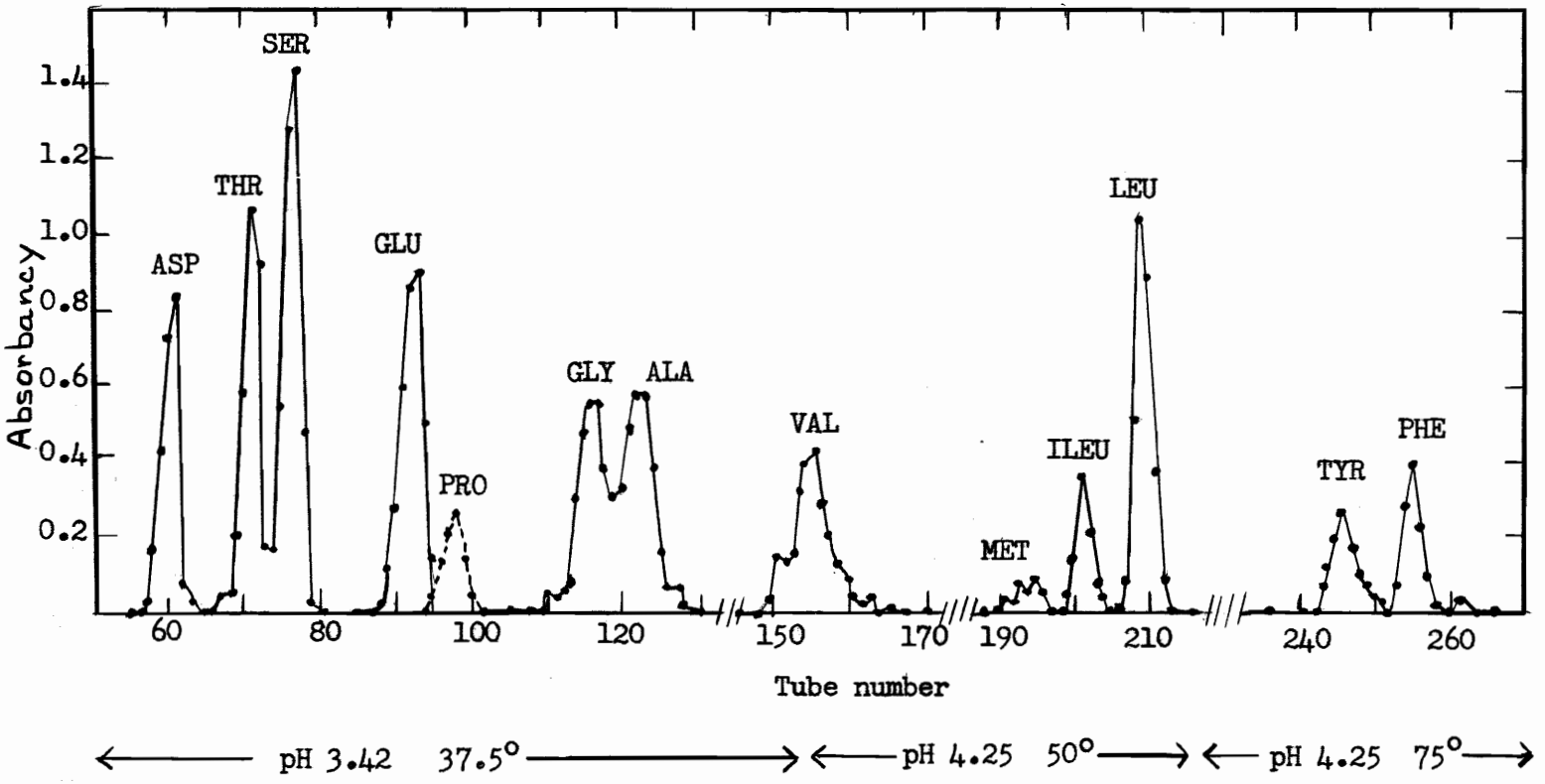


Figure 24. Elution Curves for a 20 Hour Hydrolysate of Myeloma Globulin A on a 0.9 x 100 cm. Column of Dowex 50. The ninhydrin colour yields have been corrected for base-line colours but not for the different amino acid colour yields. The absorbancy of the ninhydrin colour produced by proline was determined at 440 mμ, by the other amino acids at 570 mμ in a Coleman junior spectrophotometer. Protein hydrolysate corresponding to 1.376 mg. of protein was chromatographed for the run shown.

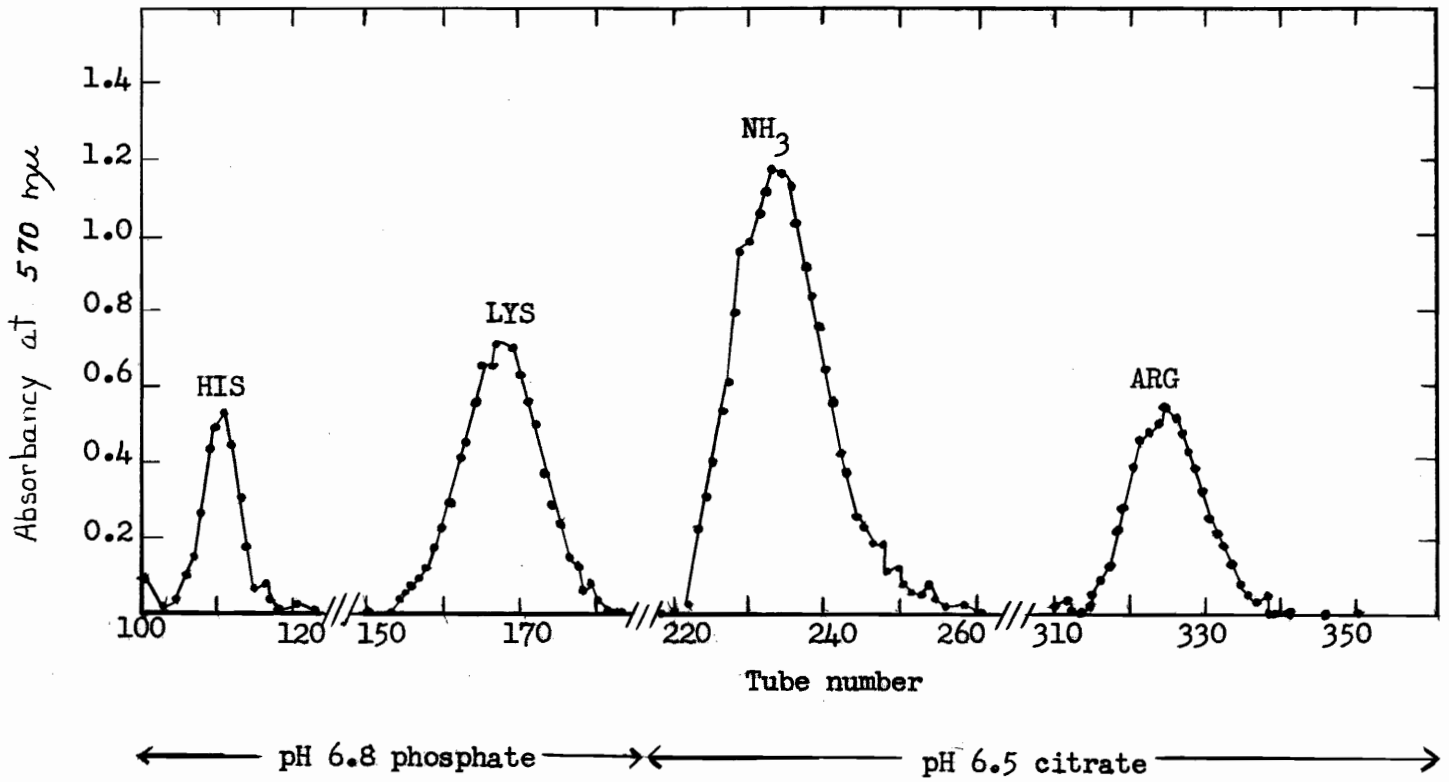


Figure 25. Elution Curves for the Basic Amino Acids and Ammonia for a 20 Hour Hydrolysate of Human Myeloma Globulin A on a 0.9 x 15 cm. Column of Dowex 50. Base-line colour has been subtracted but no correction has been applied for the colour yields of the different amino acids. The tubes containing the constituents eluted with pH 5.0 citrate buffer have been omitted from this diagram. For this run, protein hydrolysate corresponding to 7.84 mg. of protein has been used.

TABLE 31

AMINO ACID RECOVERIES FROM HUMAN MYELOMA

GLOBULIN A HYDROLYSATES

The data are presented as grams of amino acid residue per 100 grams of anhydrous, ash-free protein. The nitrogen content is taken as 16.0 per cent. Values given in parentheses are omitted from the averages. For serine, threonine and ammonia, the values cited in the column as averages were obtained by extrapolation to zero time of hydrolysis by the method of least squares.

Amino acid residue	Time of hydrolysis				Average or extrapolated value	Average deviation
	20 hour		70 hour			
Asp	7.97	7.61	--	8.27	7.95	±0.23
Thr	8.57	8.90	8.25	8.73	8.83[a]	0.20
Ser	9.38	9.35	8.85	9.40	9.46[a]	0.15
Glu	11.03	11.54	12.31	12.13	11.75	0.47
Pro	7.09	7.92	7.27	8.57	7.71	0.53
Gly	3.99	4.02	4.03	3.93	3.99	0.03
Ala	5.11	5.37	5.43	4.61	5.13	0.27
Val	(5.70)	(5.95)	7.27	7.19	7.23	0.04
Met	1.02	1.11	0.88	1.05	1.02	0.06
Ileu	2.69	2.69	2.71	2.57	2.67	4.05
Leu	9.55	9.15	10.02	9.27	9.50	0.29
Tyr	4.97	5.13	5.30	5.24	5.16	0.11
Phe	(6.13)	4.93	5.37	5.04	5.11	0.17
1/2 Cys[b]	2.51	2.65	--	--	2.58	0.07
His	2.11	2.08	2.09	--	2.09	0.01
Lys	(5.34)	(5.04)	5.51	5.57	5.54	0.03
NH_3	(1.50)	1.35	1.53	1.46	1.29[a]	0.02
Arg	5.21	4.87	5.61	5.38	5.27	0.23
Tryp[c]	--	--	--	--	1.51	±0.09

[a] These values were extrapolated to zero hours hydrolysis time by least squares.

[b] Actual recovery values.

[c] Determined colorimetrically by the method of Spies (104).

obtained by this method (5.54) agrees well with the value for lysine determined by Sanger's DNFB procedure (5.62 lysine residues per 100 gm. of protein). The content of carbohydrate has not yet been determined. This protein, however, probably contains 1 to 3 per cent carbohydrate. Table 32 lists the amino acid composition of myeloma globulin A and the nitrogen recovery. Even without a small amount of carbohydrate both the weight and the nitrogen recoveries are high. Possible reasons for this have been discussed in the preceding section. Also an incorrect assumption of the nitrogen content would influence the weight recovery but would have no effect on the per cent nitrogen recovery, since the quantity of protein in the hydrolysates was estimated from the same nitrogen content (16.0 per cent) as were the calculations of nitrogen recovery. A lower nitrogen content would lead to a weight recovery closer to 100 per cent. Both the threonine and leucine content of this abnormal globulin are significantly lower than values of these amino acids determined on normal globulin fractions (Table 1). The amino acid composition of this protein appears to be different than that of any of the myeloma proteins recently analyzed by Grisolia and Cohen (35). However, in spite of small differences in the amino acid composition of normal and abnormal γ-globulins, there exists a striking similarity.

b. Myeloma globulin B. Figure 26 and 27 are examples of typical elution chromatograms of a 70 hour hydrolysate of this protein. Tables 33 and 34 summarize the data concerning the composition of the protein. There exist significant differences between this protein and myeloma globulin A. As previously determined by end-group assay, the lysine

TABLE 32

AMINO ACID COMPOSITION OF HUMAN MYELOMA GLOBULIN A

Amino acid residue	Gm. residue per 100 gm. protein	Average per cent deviation	Gm. a.a. per 100 gm. protein	Gm. N per 100 gm. protein	N as per cent of total N
Asp	7.95	2.9	9.19	0.97	6.06
Thr	8.83[a]	2.3	10.40	1.22	7.62
Ser	9.46[a]	1.6	11.42	1.52	9.50
Glu	11.75	4.0	13.38	1.27	7.94
Pro	7.71	6.9	9.14	1.11	6.94
Gly	3.99	0.8	5.25	0.98	6.12
Ala	5.13	5.3	6.43	1.01	6.31
Val	7.23	0.6	8.54	1.02	6.38
Met	1.02	5.9	1.16	0.11	0.69
Ileu	2.67	1.9	3.10	0.33	2.06
Leu	9.50	3.1	11.01	1.18	7.38
Tyr	5.16	2.1	5.73	0.44	2.75
Phe	5.11	3.3	5.74	0.49	3.06
His	2.09	0.5	2.36	0.64	4.00
Lys	5.54	0.5	6.32	1.21	7.56
NH_3	1.29[a,b]	1.6	1.37[b]	1.13	7.06
Arg	5.27	4.4	5.88	1.89	11.81
Tryp	1.51[d]	6.0	1.66	0.23	1.44
1/2 Cys	2.58[c]	2.7	3.03	0.35	2.19
Total[e]	102.50		119.74	17.10	106.87

a These values are extrapolated values.
b These values are omitted from total.
c This value is an average of actual recovery values.
d Determined colorimetrically by the method of Spies (104).
e These totals may be incorrect because of errors made in the assumption of the nitrogen content as 16.0 per cent and in the determination of nitrogen in hydrolysates by the Kjeldahl procedure. At present an effort is being made to eliminate these errors.

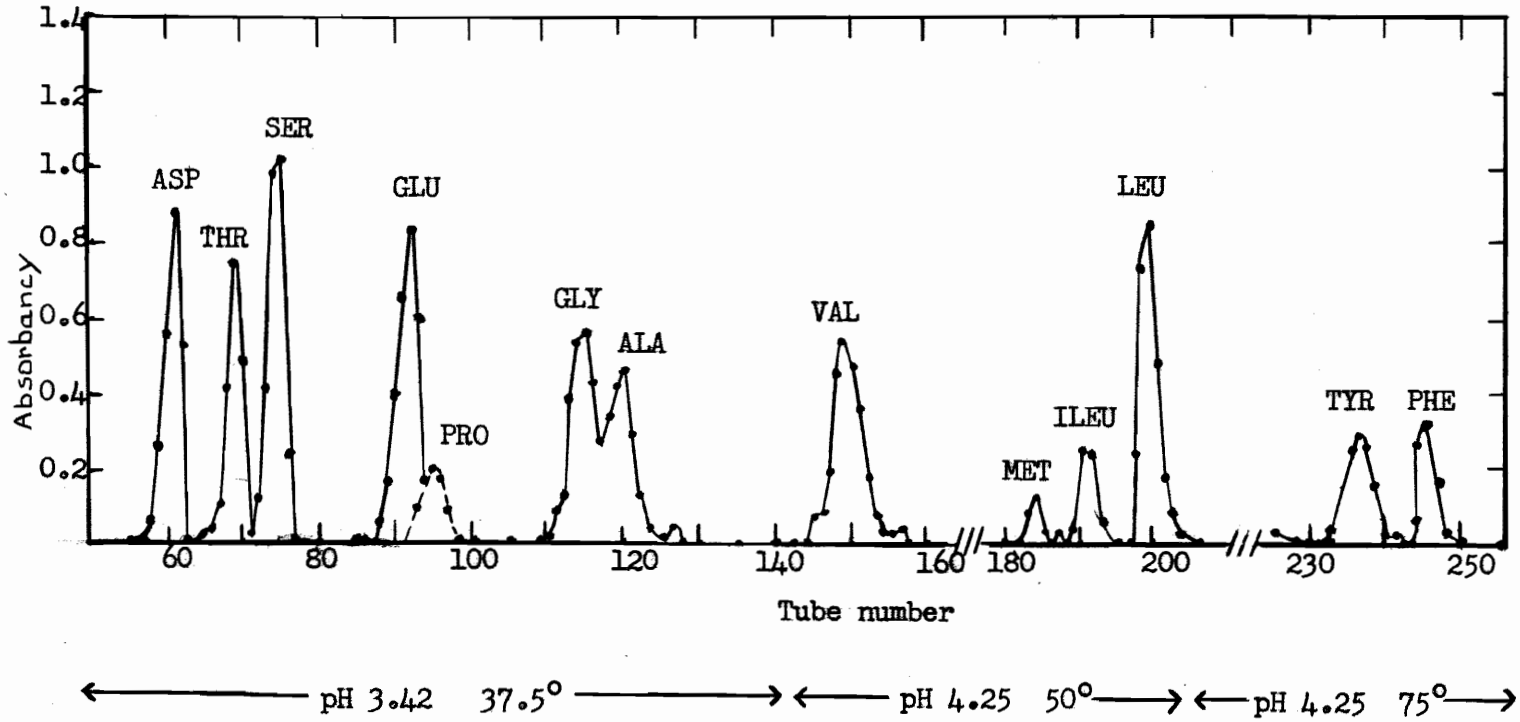


Figure 26. Elution Curves for a 70 Hour Hydrolysate of Human Myeloma Globulin B on a 0.9 x 100 cm. Column of Dowex 50. Base-line colours have been subtracted but no correction has been applied for the colour yields of the different amino acids. The proline colour was read at 440 mμ; the colours of the other amino acids at 570 mμ. For this run, 1.29 mg. of protein were used.

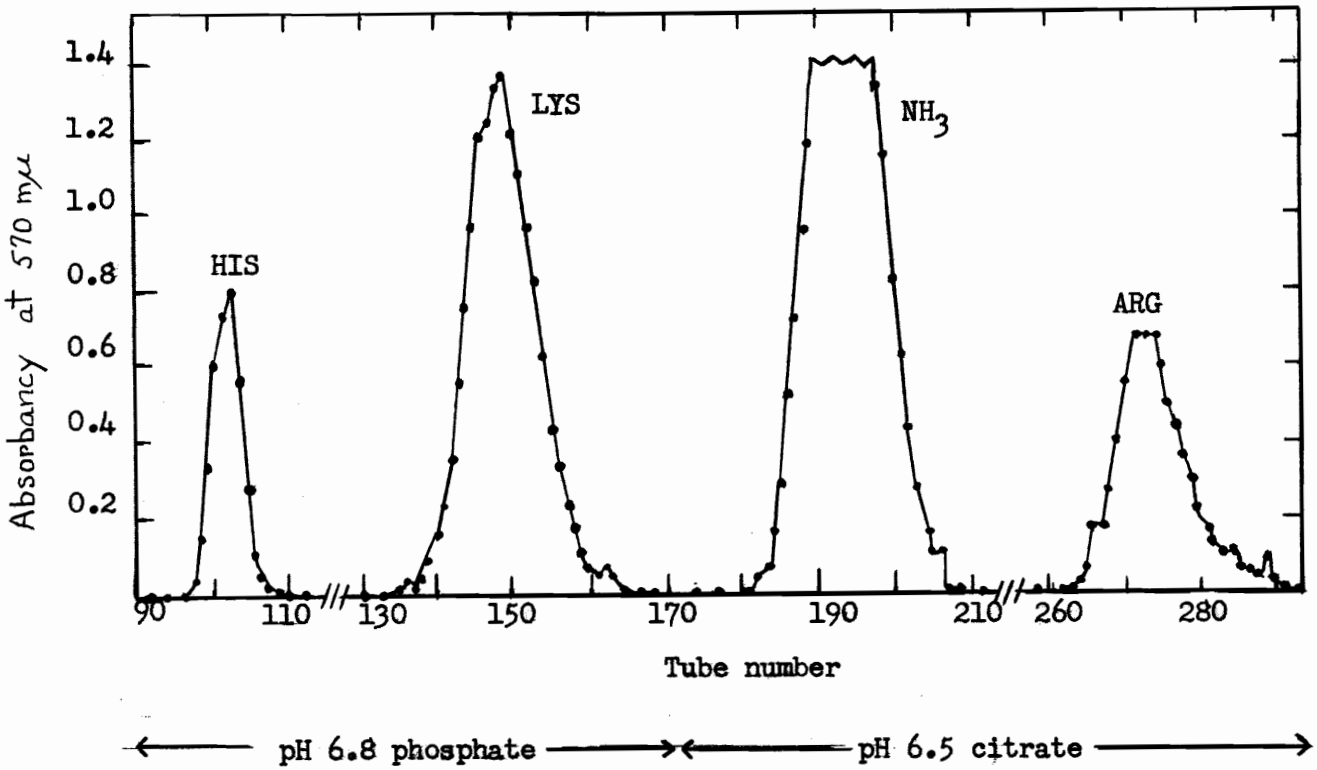


Figure 27. Elution Curves for the Basic Amino Acids and Ammonia for a 70 Hour Hydrolysate of Human Myeloma Globulin B on a 0.9 x 15 cm. Dowex 50 Column. Base-line colour has been subtracted, but no correction has been applied for the colour yields of the different constituents. For this run, hydrolysate corresponding to 8.60 mg. of protein was used. The tubes containing constituents eluted from the column by pH 5.0 citrate buffer have been omitted from this diagram.

TABLE 33

AMINO ACID RECOVERIES FROM HUMAN MYELOMA

GLOBULIN B HYDROLYSATES

The data are presented as grams of amino acid residue per 100 grams of anhydrous ash-free protein. The nitrogen content is taken as 16.0 per cent. Values given in parentheses are omitted from the averages. For serine, threonine, methionine and ammonia, the values cited in the columns as averages were obtained by extrapolation to zero time of hydrolysis by the method of least squares.

Amino acid residue	Time of hydrolysis				Average or extrapolated value	
	20 hour		70 hour			
Asp	9.32	9.14	9.21	8.97	9.16	±0.11[a]
Thr	6.87	6.63	6.65	6.56	6.81	0.08
Ser	10.05	9.99	8.00	8.12	10.80	0.05
Glu	11.22	11.06	11.76	11.68	11.43	0.29
Pro	6.28	6.44	6.46	(8.01)	6.39	0.08
Gly	3.90	3.88	4.18	4.37	4.08	0.19
Ala	4.30	4.01	4.32	4.16	4.20	0.11
Val	(6.91)	(6.81)	10.19	8.84	9.52	0.68
Met	1.42	1.31	0.96	1.12	1.50	0.07
Ileu	(2.03)	(2.20)	2.57	2.45	2.51	0.06
Leu	8.58	8.90	8.84	9.29	8.90	0.19
Tyr	7.58	6.77	7.26	7.16	7.19	0.23
Phe	6.10	5.35	5.18	5.01	5.41	0.34
1/2 Cys[c]	1.94	1.99	--	--	1.96	0.03
His	2.28	2.22	2.37	2.30	2.29	0.04
Lys	7.81	7.84	8.06	8.36	8.02	0.19
NH_3	1.31	--	1.91	1.89	1.07	0.01
Arg	4.90	5.42	5.64	5.26	5.30	0.22
Tryp[b]	--	--	--	--	1.48	0.19

[a] Average deviations.
[b] Estimated colorimetrically by the method of Spies (104).
[c] Actual recovery values of cysteic acid calculated as cystine.

TABLE 34

AMINO ACID COMPOSITION OF HUMAN MYELOMA GLOBULIN B

Amino acid residue	Gm. residue per 100 gm. protein	Average per cent deviation	Gm. component per 100 gm. protein	Gm. N per 100 gm. protein	N as per cent of total N
Asp	9.16	1.2	10.59	1.11	6.94
Thr	6.81[a]	1.2	8.02	0.94	5.88
Ser	10.80[a]	0.5	13.03	1.74	10.88
Glu	11.43	2.5	13.02	1.24	7.75
Pro	6.39	1.2	7.57	0.92	5.75
Gly	4.08	4.6	5.37	1.00	6.25
Ala	4.20	2.6	5.26	0.83	5.19
Val	9.52	7.1	11.25	1.34	8.38
Met	7.50[a]	4.7	1.70	0.16	1.00
Ileu	2.51	2.4	2.91	0.31	1.94
Leu	8.90	2.1	10.32	1.10	6.88
Tyr	7.19	3.2	7.98	0.62	3.88
Phe	5.41	6.3	6.07	0.51	3.19
1/2 Cys	1.96[c]	1.5	2.30	0.27	1.69
His	2.29	1.7	2.59	0.70	4.38
Lys	8.02	2.4	9.15	1.75	10.94
NH_3	1.07[a,b]	0.9	1.14[b]	0.94	5.88
Arg	5.30	4.2	5.91	1.90	11.88
Tryp	1.48[d]	12.8	1.62	0.22	1.38
Total[e]	106.95		124.66	17.60	110.06

[a] These values were obtained by extrapolation by the method of least squares.

[b] These values are omitted from the totals.

[c] This figure is the average of actual recoveries.

[d] Determined colorimetrically by the method of Spies (104).

[e] See footnote e, Table 32.

content of myeloma globulin B is much higher. The content of aspartic acid, serine, proline, valine and tyrosine is also quite dissimilar.

It is not unexpected that abnormal γ-globulins and normal γ-globulin fractions should vary to some extent in their amino acid content when they are compared to whole normal γ-globulin. It is well known that normal human γ-globulin is indeed a mixture of many proteins. The possibility exists that the abnormal myeloma proteins are the same as some of the normally occurring serum globulins. Immunological experiments have been reported by Kunkel et al. (51) that demonstrate that some myeloma proteins are related to at least a part of normal γ-globulin. These workers have shown that rabbit antisera against human γ-globulins II-1,2 and II-3 both reacted with the sera of four multiple myeloma patients. These sera contained abnormal γ-globulins. A fifth sera containing an abnormal β-globulin did not react with either antiserum. Adsorption of the antiserum with purified myeloma γ-globulins failed to remove all the antibody activity. Also, rabbit antisera against a myeloma protein precipitated with normal γ-globulin.

V. CONCLUSIONS

From the preceding presentation of data it is evident that different species of animals produce different kinds of γ-globulins. The rabbit, normal or immune, produces only one type of γ-globulin molecule, a single polypeptide chain. The human, on the other hand, manufactures a number of different γ-globulin molecules, some of which appear to be composed of two or three polypeptide chains. It is noteworthy that although human γ-globulin is definitely a mixture of proteins, the majority of these proteins have aspartic or glutamic acid, or both, as their N-terminal residues. Even three abnormal human γ-globulins studied possessed one or both of these N-terminal residues. Bovine and equine serum globulins are also a mixture of many proteins. They differ from human γ-globulins, however, being mixtures of proteins with a great diversity of N-terminal amino acid residues.

What do these facts mean with respect to the formation of antibodies? It is, perhaps, wisest to consider first the formation of rabbit antibodies which do not differ chemically from normal rabbit γ-globulin and later discuss the apparently more complex situation of antibody formation occurring in the human, horse and cow.

A. Rabbit Antibodies and γ-Globulins

Antibodies produced in rabbits to various antigens have been found to be indistinguishable by ultracentrifugal and electrophoretic studies from normal rabbit γ-globulin. Four different rabbit antibodies have been shown to be of the same amino acid composition and to possess the same N-terminal sequence of amino acid residues. As pointed out by Porter (80), 19^5 pentapeptides could theoretically occupy the terminal position of a molecule the size of rabbit γ-globulin. Therefore, it does not seem unreasonable to assume that the complete sequence of amino acids in all rabbit γ-globulin molecules is the same. This assumption will be even more valid if it is possible to demonstrate that the C-terminal sequence of normal and antibody globulin does not differ. If rabbit antibodies are chemically identical with rabbit γ-globulin, the serological distinction of antibodies can only be due to certain variations in the configuration of the normal globulin molecule. It is entirely possible, of course, that the specificity of antibodies is due not to the protein portion of the molecule but to the antibody carbohydrate. The only experiments which can be counted as evidence in this direction were performed by Peterman and Pappenheimer (77) who showed that after diphtheria antitoxin had been digested with pepsin, the fraction still possessing antitoxic activity was nearly 4 per cent carbohydrate. Northrop (67) later showed that crystalline diphtheria antitoxin contained only 2 per cent carbohydrate. In this present discussion, however, the role of carbohydrate in antibody specificity will not be considered. The experiments of Heidelberger *et al.* (44) as well as the

work of Green and Anker on the incorporation of labelled amino acids into antibody and serum proteins conclusively shows that γ-globulin in the serum is not unfolded and then refolded to produce antibody. Therefore, it seems logical to accept Pauling's (74) hypothesis that normal and immune γ-globulins are formed from the same uncoiled polypeptide precursor, and in the presence of antigen the uncoiled chain assumes a folded structure that is complementary in shape to the antigenic determinant groups.

Antibody synthesis, like any type of protein synthesis, probably occurs as a two-phase process. In the first phase, free amino acids or peptides combine to form a long polypeptide chain. Necessarily, an enzyme system must overcome the activation energy needed for the synthesis of the peptide bonds. Haurowitz (37) does not attribute the specific order of the amino acids to the action of an enzyme system but rather to the existence of a template or "organizer". The second step in the synthesis of antibody or normal globulin concerns the folding of the polypeptide chain into a globular protein molecule. An antigen would, therefore, appear to influence only the second phase of this process. This influence could be due to the polar groupings present in all known antigenic substances. The electrostatic forces of these groups could act on adjacent amino acid residues in the uncoiled or partially coiled polypeptide chain thus determining the ultimate configuration of that part of the molecule.

All these postulations necessitate the persistence of antigen, or at least of antigenic determinant groups, within the cell throughout the period of antibody production. Although there is no direct evidence for

this, especially in cases of life-time immunity, it is possible that the failure of investigators to detect the presence of antigenic agents is due only to the insensitivity of the available analytical techniques. On the other hand, Libby (54) has shown that when antigens labelled with radioactive atoms have disappeared from the host, the production of antibodies to those antigens is no longer detectable.

Before the question of antibody formation in the absence of antigen is considered, an analysis of the data obtained concerning γ-globulins of species other than rabbit should be performed.

B. Equine and Bovine Antibodies and γ-Globulins

End group analysis has proven what physical and immunological studies had indicated; normal and immune equine and bovine serum globulins are mixtures of many different proteins. Whether these proteins differ by more than one or two amino acid residues cannot at present be determined. The existence of many proteins, at first thought, may seem to contribute evidence against a theory of antibody formation involving merely the folding of a preformed polypeptide chain. Each protein in the heterogeneous mixture could possibly be a single type of antibody that had been synthesized under the influence of its respective antigen. This idea loses credibility with the knowledge that a specifically precipitated equine antibody is also a mixture of many proteins. A more plausible explanation can be derived by assuming that the horse normally produces many similar serum globulins. One or many fairly long peptide sequences may be common to all these proteins. Under the influence of the polar groups of an antigen,

any or all of these types of molecules in their unfolded form may assume a configuration complementary to the determinant group on the antigen. Such an hypothesis would also explain why certain antibodies, for example, tetanus antitoxin, occur in equine T-, β-, and γ-globulin fractions. Some proteins in each of these fractions apparently possess a suitable amino acid sequence for the production of a configuration of complementary shape and charge distribution to the antigenic determinant group. Equine anti-pneumococcal antibodies, however, occur only in the γ-globulin fraction suggesting that only the γ-globulins are capable of assuming the required spatial configurations. Of course, it is possible that if different body cells are responsible for the synthesis of different globulins, only the γ-globulin-producing cells are capable of "trapping" these pneumococcal antigens.

The observed multiplicity of antibodies to a single antigen can also be due to the fact that antibodies are complementarily adapted to different portions of the antigen molecule. As was pointed out earlier, Cann (17) has separated by electrophoresis convection, two different antibodies to bovine globulin-azophenylarsonate. It is also reasonable to believe that certain globulins are capable of forming better antibodies than others. Haurowitz (36) believes that all the "intermediates between normal serum globulins and well adapted antibodies are present in immune sera". Figure 28 presents Haurowitz (36) idea of well adapted, poorly adapted and ideal antibodies.

It is not too surprising that the quantitative spectrum of DNP-amino acids recovered from a specific equine antibody differs from that of

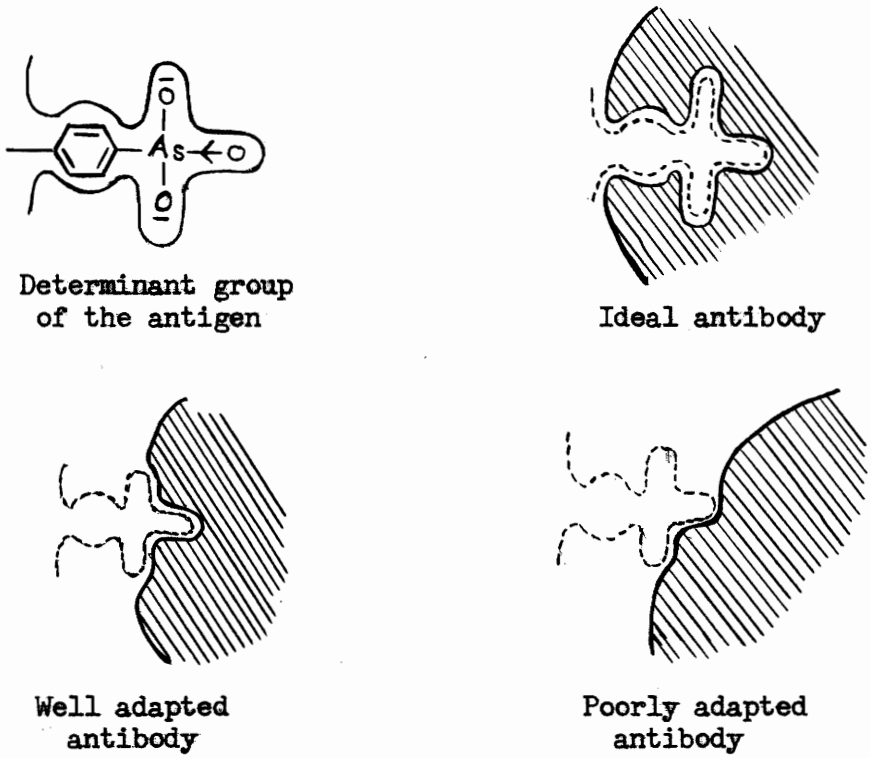


Figure 28. Types of Antibodies Formed to a Phenylarsonic Acid Group. These drawings are copied from Haurowitz (36).

whole equine γ-globulin. In the case of the equine antibody against Type III pneumococcal polysaccharide, γ-globulin precursors with N-terminal alanine residues are apparently preferentially or most easily adapted to the shape of the determinant group or groups on the antigen.

Because of the limited investigation of bovine serum globulins, little can be said. Normal, as well as hyperimmune bovine γ-globulin is a mixture of many proteins. Only conjectures can be made concerning the

nature of single bovine antibodies. It seems probable, however, that bovine, like equine antibodies will also be found to be mixtures of many proteins.

C. Human γ-Globulins

Aspartic and glutamic acid were the only amino acids found to be N-terminal residues of normal and abnormal γ-globulins. Putnam (83) has reported the occurrence of a myeloma γ-globulin with neither of these amino acids in the N-terminal positions. These end-group determinations prove that normal human γ-globulin is a mixture of several different molecular species. They also show that some of the molecules are composed of two or more polypeptide chains. The human multiple myeloma globulins analyzed to date, with the exception of cryoglobulin B, appear to be homogeneous. Cryoglobulin B, however, is certainly a mixture of at least two proteins.

It would appear that myeloma patients profusely synthesize only one or two of the normally occurring molecules. (Multiple myeloma patients have been known to have serum proteins that were 52 per cent γ-globulin. Normal serum protein is about 12 per cent γ-globulin.) Until an improved method of chromatography or electrophoresis makes possible the separation of human γ-globulin into its constituent proteins, it will be impossible to decide if the abnormal γ-globulins found in the sera of patients with multiple myeloma are also found in smaller quantities in normal sera.

Because there is some evidence that antibodies are produced by plasma cells or their precursors (28,29), and because myeloma proteins are produced during the rapid proliferation of these cells, there may

possibly be some relation between these two types of proteins. In this disease the plasma cells apparently are influenced in some way that results in the production of great amounts of one or two globulins. Could an antigen also stimulate these cells to produce one globulin, a specific antibody? It would be interesting to determine whether or not a specific antibody from human immune sera is a single protein. More than likely, however, the same situation would be found for the human as was observed for the horse.

D. The Formation of Antibodies

In the foregoing discussion, data have been presented in support of a theory of antibody production proposed by Pauling (74). This theory assumes that antigens influence the folding of the globulin molecule thus causing the production of specific antibodies. Burnet and Fenner (12) have objected to this theory on the grounds that antibody production is known to continue when antigen can no longer be detected in the body. This objection has been discussed in Section V-A. They also believe that the theory of Pauling cannot explain the phenomenon of the accelerated secondary response to the antigen. This objection is questionable, however. It is possible that cells already producing antibody and therefore, containing it, may be better capable of "trapping" the specific antigen.

It has definitely been established that normal rabbit γ-globulin and antibody globulin do not differ. Therefore, in the case of antibody formation in the rabbit, the antigen-modified enzymes or "adaptive

enzymes" postulated by Burnet and Fenner (12) must influence only the mode of folding or coiling of the γ-globulin precursor. Whether the antigen itself or an antigen-produced substance controls the folding and spatial configuration of an antibody cannot be decided.

The basic weakness in Burnet's theory would seem to be the fact that there is no evidence for enzyme adaptation of this kind in other fields. Although the occurrence of "adaptive enzymes" has been reported by Spiegelman (101,102,103) and others, the substrates of all these "adaptive enzymes" are naturally occurring compounds closely related in structure to the substrate of a normally occurring enzyme. It seems hard to imagine that an antigen could be able to modify an enzyme to produce antibodies against such compounds as arsanilic acid, or other synthetic dyes.

The data obtained in this research support Pauling's theory of antibody formation. However, they do not disprove Burnet's theory if it is interpreted to mean that an antigen does not initiate the production of a chemically different protein but merely modifies an enzyme system to produce a globulin that is folded in a new way. If all protein synthesis is considered to occur in two steps, first, the production of an uncoiled polypeptide chain and secondly, the folding of this chain into a stable configuration, Burnet's "adaptive enzymes" must influence only the second step.

The decision of whether the antigen itself influences the configuration of its specific antibody as Pauling believes or the antigen stimulates the formation of another molecule for this purpose as Burnet postulates can not be made on the basis of the chemistry of antibodies.

VI. SUMMARY

1. Eight immune rabbit γ-globulins and three abnormal human myeloma γ-globulins have been characterized by electrophoretic and ultracentrifugal studies.

2. Eight specific pneumococcal polysaccharide-rabbit antibody precipitates as well as normal and hyperimmune rabbit γ-globulins were prepared.

3. The 2,4-dinitrofluorobenzene technique described by Sanger (86) was used to determine the free amino groups of several rabbit, human, bovine and equine serum globulin preparations.

All the rabbit γ-globulins possess one N-terminal alanine residue and 70 lysine residues. DNP-aspartic acid isolated from every hydrolysate of DNP-rabbit γ-globulin suggests the presence of a labile bond involving the amino group of an aspartyl residue.

Human γ-globulin II-1,2 has one N-terminal aspartic acid residue and two N-terminal glutamic acid residues; human γ-globulin II-3, one aspartic and one glutamic acid as N-terminal residues. These findings suggest that human γ-globulin is a mixture of several different proteins, some composed of two or more polypeptide chains. The two γ-globulins isolated from the sera of multiple myeloma patients each has two N-

terminal glutamic acids. The lysine content of these proteins, however, differs. A purified cryoglobulin has approximately 1.5 moles each of N-terminal aspartic and glutamic acid residues indicating that it also is a mixture of at least two γ-globulins.

The hydrolysates of DNP-derivatives of bovine and equine serum globulins as well as a specifically precipitated equine antibody contained several DNP-amino acids each present in less than a molar quantity. This suggests that bovine and equine serum globulin like human γ-globulin is a mixture of many proteins which differ in the nature of their N-terminal residues.

4. The N-terminal sequence of amino acid residues in four specific rabbit antibodies was determined by methods described by Sanger (87) and Porter (80,82). The sequence alanylleucylvalylaspartylglutamyl which occurs in rabbit antiovalbumin (80) and normal rabbit γ-globulin occurs in each of these antibodies suggesting that only one species of γ-globulin is produced by the rabbit.

5. Amino acids are hydrolyzed from human and rabbit γ-globulins by carboxypeptidase. These proteins, therefore, apparently have one or more free α-carboxyl groups.

6. The amino acid composition of four rabbit antibodies and two human myeloma γ-globulins has been determined mainly by chromatography of hydrolysates of the proteins on columns of Dowex 50 (62). No difference in the composition of the rabbit antibodies was found. The abnormal human γ-globu-

lins differ significantly from each other and from normal human γ-globulin.

7. The significance of these data has been discussed in terms of the current theories of antibody formation.

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